Biogeography and Incipient Speciation of the Acorn Barnacles, *Tetraclita* species in NW Pacific

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A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of Master of Philosophy

in

Biology

July 2007

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Declaration

I declare that this thesis represents my own works and that it has not been previously included in a thesis, dissertation or degree, diploma or other qualification.

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Abstract

Barnacles are dominant space occupiers in intertidal rocky shore and play an important role in its ecological assemblage. Species from the genus *Tetraclita* are common in East Asia and three species, *Tetraclita squamosa*, *T. japonica* and *T. formosana* have been described. They can be easily distinguished by parietes colour of green, grey to purplish-grey, and pink respectively. However, the taxonomic status of the three species is controversial and their population structure is poorly understood.

In the present study, genetic differentiation among the three *Tetraclita* species was studied using DNA sequence data from mitochondrial cytochrome *c* oxidase subunit I (COI), 12S rRNA and nuclear internal transcribed spacer 1 (ITS1) to evaluate their taxonomy. The population structure of *T. japonic*a and *T. formosana* was also examined using mitochondrial control region sequences and amplified fragment length polymorphism (AFLP) to investigate their evolutionary history in northwestern Pacific.

The molecular analysis indicates that *Tetraclita squamosa* occurs in two genetically distinct forms in East Asia. The NW Pacific form (Japan, Okinawa and Taiwan) differ from the South China form (Xiamen and Hong Kong) by 15-16% in COI divergence, which is comparable to values for other congeneric barnacle species. The 12S rRNA and ITS1 sequences are also distinct between the two forms. These results suggest that the two forms represent two distinct species with the NW Pacific form being a new species. The larval supply of the NW Pacific is associated with the

Kuroshio Current while the larva of S China form is transported by South China Sea Current in summer. Comparing sequences from this study to those obtained from other studies show that "*T. squamosa*" from Australia and Singapore are also genetically distinct from the two forms in this study indicating that "*T. squamosa*" probably consists of a cryptic species complex in its distribution range.

While *Tetraclita japonica* is widely distributed in Japan and southern China, *T. formosana* is found almost exclusively in Taiwan. They are similar in morphology which leads to uncertainty in their species status. In the present study, I found no significant genetic differentiation among *Tetraclita formosana* and the two color forms (grey and purplish-grey) of *T. japonica* in COI and control region. The two taxa also share common nuclear ITS1 genotypes, providing no evidence for reproductive isolation. The two taxa only differ in the color of the parietes and geographical distribution leading to the question whether the two taxa represent two color morphotypes or unsorted lineages. AFLP was therefore applied to study the variation in a large number of independent nuclear loci among populations of the two taxa.

The genetic differentiation between *T. japonica* and *T. formosana* was investigated using 209 AFLP markers and 341 individuals from 12 locations. The two taxa are genetically highly differentiated (Φ_{CT}=0.267). Bayesian analysis and principal component analysis indicate the presence of hybrids in *T. formosana* samples from Japan. Strong differentiation between northern (Japan, including Okinawa) and southern (southern China) populations of *T. japonica* is revealed supporting the result from mtDNA control sequence analysis. The lack of mtDNA

divergence between the two taxa suggests they diverged recently, probably in allopatric glaciation refugia during the Pleistocene. Hybridization may occur in contact zone following glacial retreat and subsequent range expansion. The genetic differentiation is maintained by oceanographic pattern that restricts larval dispersal. This is supported by population structuring observed within the two taxa. Our results provides new insights on the effect of glaciation in promoting speciation in NW Pacific and how the combination of historic vicariance and contemporary dispersal pattern shape the genetic structure and distribution of intertidal biota.

This thesis research demonstrates that "*T. squamosa*" constitutes a cryptic species complex instead of a single cosmopolitan species. Further studies are needed to confirm the identity and geographical distribution of the 'widely distributed' *Tetraclita squamosa* in the Indo-West Pacific. In contrast, although *T. japonica* and *T. formosana* are genetically differentiated based on AFLP analysis, occurrence of hybridization and lack of mtDNA divergence between the two taxa suggest that they probably represent two subspecies rather than distinct species. The results also provide new insights on the biogeography of the NW Pacific region.

摘要

簕壺是潮間帶岩岸的主要生物，牠們對該處的生態結構起著重要的角色。笠簕壺(笠簕壺屬 *Tetraclita*)是東亞地區常見的簕壺，現今有三個品種可以在這區找到，分別是鱗笠簕壺(*Tetraclita squamosa*)，日本笠簕壺(*T. japonica*)以及台灣美麗笠簕壺(*T. formosana*)。牠們可以藉著不同的外殼顏色來分辨。牠們的外殼顏色分別爲綠色、灰至紫灰色、以及粉紅色。可是這三種簕壺的分類地位仍存在許多爭議，而我們對牠們的族群結構亦知之甚少。

在本研究中，我會利用綫體細胞色素氧化酶 I 亞基(COI)和 12S rRNA 以及細胞核轉錄間隔區(ITS)1 的基因序列來研究三者的遺傳分化，以闡明牠們的分類，同時亦會利用結粒體控制區域的基因序列和擴增片段長度多態性(AFLP)來調查日本笠簕壺和台灣美麗笠簕壺的族群結構，用以研究牠們在西北太平洋地區的進化歷史。

分子分析顯示鱗笠簕壺在東亞洲地區存在著兩個遺傳上顯著不同的形。西北太平洋形（日本，沖繩及台灣)和南中國形（廈門及香港)在 COI 的基因序列中有著 15-16%的差異，這個差異與其他同屬簕壺品種之間的差異相約，兩種形的 12S rRNA 和 ITS 1 的基因序列亦存明顯的差異。這些結果顯示這兩個形分別代表著兩個不同的品種，而西北太平洋形應該爲一個新品種。西北太平洋形的幼體應透過黑流供應，而南中國形的幼體應在夏天經由南中國海海流所傳送。當我們將這次研究獲得的基因序列和其他研究所得的作比較，發現澳洲以及星加坡的 "鱗笠簕壺" 亦和是次研究的兩個形有着明顯的差異，這顯示鱗笠簕壺在其分佈區域中存在着一個隱藏的物種群組。

日本笠簕壺於日本以及南中國地區廣泛分佈，而台灣美麗笠簕壺則主要分佈於台灣，牠們的形態非常相似，以致難於界定牠們是否不同的品種。在本研究中，在 COI 和結粒體控制區域基因序列中，台灣美麗笠簕壺以及日本笠簕壺的兩個顏色型〈灰色及紫灰色〉之間沒有顯著的遺傳分化，而牠們也擁有共同的基因型於 ITS 1 基因座，顯示沒有證據支持兩者之間存在生育隔離，牠們只

於外壺顏色以及地埋分佈上有不同，所以對於兩者應爲兩個顏色型還是未完成譜系排序的相近種仍然存疑，故此，我運用了 AFLP 技術於兩種物種的族群上，以調查大量獨立細胞核的基因座中的差異。

利用 209 個 AFLP 標記，341 個從 12 個地區而來的台灣美麗笠籐壺以及日本笠籐壺個體之間的遺傳分化被分析了，結果顯示兩個物種之間有着顯著分化。貝葉斯分析及主要分分析顯示，日本的台灣美麗笠籐壺樣本中存有雜種，而日本藤壺的北方〈日本〉與南方〈南中國〉族群之間亦存有強烈分化，這個結果與線粒體控制區基因序列所得到的吻合。兩個物種之間基因序列缺乏差異，表示牠們的分化發生於不久之前，可能於更新紀冰河時期的異域性避難所。當冰河退卻後，牠們的分佈範圍擴張，並且於接觸帶發生雜交，海洋水流的模式限制了幼體的散播和兩者之間的基因流，從而維持兩者之間的分化，兩個品種中的存在的族群結構支持了這一論點。這些結果爲我們提供了對冰河時期如何促進西北太平洋地區的物種形成的新理解，以及歷史性散離與當代的散播模式對潮間帶生物的遺傳結構和分佈的影響之認識。

這個論文研究示了「鱗笠藤壺」構成了一個隱藏物種族群而非單一廣泛分佈的物種，我們需要進一步的研究去確認這個「廣泛分怖」的鱗笠藤壺的身份與及其於印度西太平洋內的地理分佈。另一方面，日本藤壺和台灣藤壺雖然在 AFLP 分析中顯示出遺傳分化，但由於牠們之間有雜交的發生，以及在線粒體基因中並沒有存在差異，因此，牠們應該作爲兩個亞種而非兩個獨立的種，這些結果亦爲西北太平洋地區的生物地理學提供了新的認識。

Acknowledgements

I sincerely thank my supervisor, Prof Ka Hou Chu for his advice and encouragement during these two years of MPhil study. I am grateful to have these invaluable experiences in my life. I am glad to receive the guidance from my thesis committee members, Prof Liwen Jiang and Prof Chong Kim Wong during my thesis research. It is my honor to have Prof. Clifford Cunningham (Duke University, USA) as my external examiner who provided valuable comments to the draft of this thesis.

My research works would not be possible without the kindest support from Dr Benny Kwok Kan Chan (Academia Sinica) for specimen collection, discussions on project direction and comments in early drafts of this thesis. I would also like to express my appreciation to Prof Gray Williams, Dr Wai Chuen Ng and Ms Damgy Hoi Lam Chan (The University of Hong Kong) for their advice and assistance in my research project, and to those provided help in field work and samples collection, including Mr A Murata and Dr F Hironobu (Seto Marine Laboratory, Kyoto University, Japan), Prof M Yamaguchi (Univ. of Ryukyus, Okinawa), Dr Min Liu (The University of Hong Kong), and Dr Kwang-Sik Choi (Cheju National University, Korea). I also thank the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR Government for permission to collect *Tetraclita* in the Cape d'Aguilar Marine Reserve.

I also thank my colleagues and technicians in the Simon FS Li Marine Science Laboratory of the Chinese University of Hong Kong for their assistance and support, in particular Ms KY Ma and Ms TH Wu for technical assistance.

Finally, I would like to thank my family for their support during my two years of postgraduate study.

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Chapter 1

General introduction

Acorn barnacles are common space occupiers in the intertidal zone of tropical and sub-tropical waters (Newman & Ross 1976) and play an important role in the ecology of intertidal assemblages (see Reimer 1976a, b; Bertness et al. 1998). Despite their ecological importance, taxonomic confusion caused by variations in external features has retarded our understanding of their ecology and biology, particularly for some common, widely distributed species such as *Chthamalus malayensis*, *Chthamalus moro* (Southward and Newman, 2003) and the *Tetraclita squamosa* subspecies complex (Darwin, 1854; Newman and Ross, 1976). Additional criteria are, therefore, needed to resolve the taxonomic confusion in these taxa.

In the NW Pacific region, members of the genus *Tetraclita* are dominant. Presently, at least 20 *Tetraclita* species have been identified (Ross 1969, 1999; Newman & Ross 1976). *Tetraclita squamosa* is the most widely distributed species of the genus throughout the Indo-West Pacific region and is thought to constitute a subspecies complex due to great morphological variations (Newman & Ross 1976). In East Asia, three subspecies, *T. squamosa squamosa*, *T. s. japonica* and *T. s. formosana* were originally described (Bruguière 1789; Pilsbry 1916; Hiro 1939). Later on, Yamaguchi (1987) and Hasegawa et al. (1996) considered *T. s. japonica* and *T. s. formosana* to be two distinct species, based on allozyme analysis and DNA sequences of mitochondrial cytochrome *c* oxidase I (COI), respectively. Thus, the three subspecies were recognized as distinct species, namely *T. squamosa*, *T.*

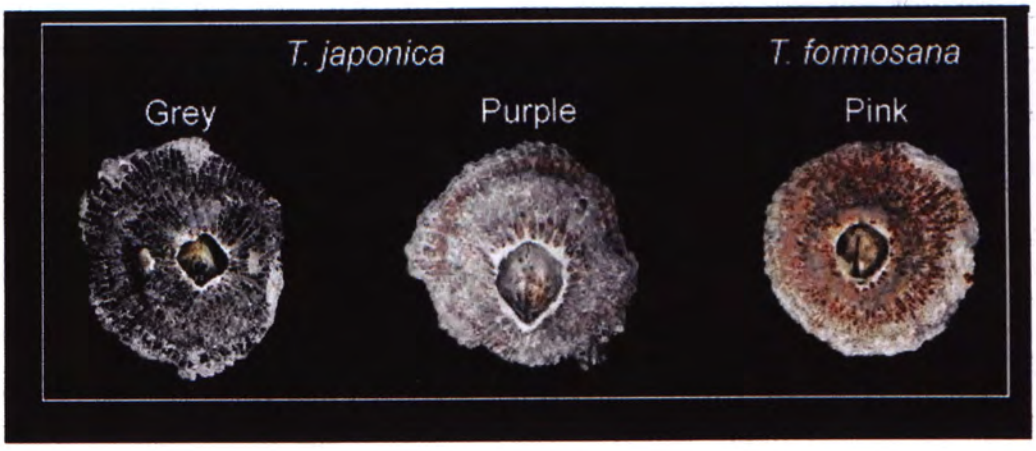
japonica and *T. formosana* (Yamaguchi 1987). Subsequent studies on adult and larval morphology of *T. squamosa* and *T. japonica* also found differences in tergo-scutal plates shape and sculpture on the larvae (Chan 2001, 2003) supporting the view that *T. squamosa* and T. *japonica* represent distinct species.

Yet the morphological and genetic differentiation between *T. japonica* and *T. formosana* is minimal. In the study by Yamaguchi (1987), only malate dehydrogenase out of 11 enzymes studied showed significant allele frequency differences and no fixed allele was found in either taxon. Moreover, the samples used for allozyme analysis were collected from a single locality so that the data provide little evidence on reproductive isolation of the two taxa as the difference in allele frequency in a single locus could be an artifact of random sampling. In the study by Hasegawa et al. (1996), the COI sequence divergence between *T. japonica* and *T. formosana* was found to be 1.2% (compared to ~19% between *T. squamosa* and the two taxa). Yet many congeneric barnacle species exhibit high COI divergence, e.g., >14% in *Chthamalus* and >9% in *Euraphia* (Wares 2001), while intra-specific divergence can be up to 2% (Sotka et al. 2004; Zardus and Hadfield 2005). *T. japonica* and *T. formosana* are also morphologically very similar; the only diagnostic difference is that *T. japonica* has grey parietes (the shell of barnacle) and individuals with purplish-grey parietes were also reported (Fig 1.1A) whilst parietes color of *T. formosana* is pink (Hiro, 1939; Fig 1.1A). They also differ in geographical distribution. In Japan, both *T. japonica* and *T. formosana* are reported, whereas in Taiwan, *T. s. formosana* exists in high abundance and almost no *T. japonica* is found (Hiro, 1939; Yamaguchi, 1987). Thus, whether the two taxa represent distinct species remains uncertain.

On the other hand, *Tetraclita squamosa* is characterized by its green parietes (Fig. 1.1B) yet there are reports on variation in morphology and zonation patterns in *T. squamosa* from different geographical regions in East Asia, suggesting the possibility of cryptic species in this widely distributed species. Pilsbry (1916) reported that the tergum of *T. squamosa* from Japan, as illustrated by Krüger (1911) was different from that of *T. squamosa* collected from China and the Philippines. Moreover, *T. squamosa* occurs in the mid shore with *T. japonica* in Japan (Yamaguchi 1987), whilst in southern China, *T. squamosa* is a low shore inhabitant, separated from *T. japonica* which is found on the mid shore (Ren & Liu 1979; Chan 2001). Whether these morphological and ecological differences in *T. squamosa* are induced by environmental factors or actually represent cryptic genetic difference is obscure.

Elucidating the taxonomic status and genetic differentiation among populations of these three *Tetraclita* taxa (see Table 1.1 for a summary on the colour and distribution of the three species) not only provides a future framework in ecological studies, but also enhances our understanding of the biogeography of the NW Pacific biota and phylogeny of this ecologically important genus. In my thesis research, I investigated the genetic differentiation among different populations of these three species in the NW Pacific region to elucidate their taxonomic status using both nuclear and mitochondrial DNA markers. Moreover, amplified fragment length polymorphism (AFLP) was also employed to study the phylogeography of the recently diverged *T. japonica* and *T. formosana*.

A.



B.

Fig. 1.1A Color variation in the external parietes of *T. japonica* and *T. formosana*. B. Natural view of *T. squamosa* on the shore.

Table 1.1 A comparison among the three *Tetraclita* species in East Asia.

	T. squamosa	*T. japonica*	*T. formosana*
Parietes color	Green	Grey to purplish-grey	Pink
Distribution	Indo-West Pacific	South China and Japan	Taiwan, Okinawa and Japan

Chapter 2

Literature Review

2.1 Molecular systematics

Species are the fundamental unit in any biological research. The literature about species concept might be more extensive than any other subjects in evolutionary biology. In spite of the seemingly endless debates over different species concepts, evolutionary biologists have increasingly shifted their interests to empirically delimiting species as a practical issue. With the advent of relatively rapid and inexpensive DNA sequencing techniques, biologists could resolve taxonomic confusion (Appelbaum et al. 2002; Tsukaya et al. 2003) and identify cryptic species (Knowlton 2000; Bickford et al. 2007) which are difficult to delineate purely on a morphological basis. The concept of a "DNA barcode" has also been proposed, which uses a fragment of DNA sequence to diagnose a species (Hebert et al. 2003; Blaxter et al. 2005). Although the practical applicability of such DNA barcoding remains controversial (Moritz & Cicero 2004; Meier et al. 2006), it is little doubt that molecular methods have provided effective tools for systematics. The development of a novel statistical framework such as nested clade analysis (NCA; Templeton 2001) and tree based methods combined with morphology (Wien & Penkrot 2002) facilitate the analyses on molecular data which can help us to delimit species, an important part of understanding evolutionary mechanisms and processes.

At the population level, the demographic structure within a species is usually very extensive and diverse and this intraspecific structure reflects both

historic vicariance and contemporary dispersal events. Phylogeography is the study concerning the principles and processes governing the geographic distributions of genealogical lineages (Avise 2000). Most of these phylogeographic analyses are fueled by advances in molecular techniques, especially the analysis of mtDNA, and other PCR-based techniques such as AFLP and microsatellites. Thus, genetic analyses have provided effective means for exploring the dynamic between micro- and macroevolution disciplines and revealing how different ecological and geographical factors shape the present biotic pattern (e.g. Wares & Cunningham 2001; Marko 2004).

In this review, I will discuss several examples of the usage of molecular methods in identifying cryptic species, delineating species, morphotypes and hybrids, and elucidating phylogeography of organisms in relation to the Pleistocene glaciation, with a major focus on the marine biota.

2.2 Molecular markers as a tool for identification of cryptic species

Many biologists may regard cryptic species as a synonym for "sibling species" (Saez & Lozano 2005) and some may even imply a sister species relationship (Knowlton 1993). I only restrict the definition of cryptic species as two or more true species that are currently classified as a single species.

Most cryptic species are morphologically so similar that are nearly indistinguishable. Cryptic species are present in most types of habitat and across different phyla, from the deep sea (Vrijenhoek et al. 1994) to tropical rain forests

(Hebert et al.. 2004), and from invisible pathogens (Koufopanou et al. 2001), small insects (Hebert et al. 2004) to mammals (Ravaoarimanana et al. 2004). Knowlton (1993) summarized the literature showing the prevalence of cryptic species (termed "sibling species" in the review) in marine habitats. She also reviewed the application of genetic data to delimit the species boundaries for these diverse arrays of marine organisms (Knowlton 2000). Since then, reports on the discovery of cryptic species by molecular technique grew exponentially (reviewed in Bickford et al. 2007)

Many of these cryptic species are genetically diverged in spite of a high degree of morphological similarity suggesting that morphological stasis or similar ecological requirements (Knowlton 2000). The snapping shrimp from genera *Alpheus* and *Synalpheus* are one of the well documented examples of cryptic species complexes in the marine environment (Knowlton & Keller 1985; Duffy 1996; Williams et al. 2001). In addition to the lack of morphological differentiation, many snapping shrimp cryptic species show no ecological specialization and occur in sympatry (Knowlton & Keller 1985; Mattews et al. 2002). Thus, cryptic speciation is suspected to be "non-adaptive" with allopatric isolation followed by secondary contact (Mattews 2006). Mattews (2006) investigated the genetic differentiation of the *Alpheus armillatus* species complex in Caribbean using mitochondrial 16S rRNA and nuclear myosin heavy chain gene sequences. She discovered four lineages with overlapping distribution and demographic analyses indicated rapid population expansion within all of the four lineages. This suggested that these *Alpheus* species derived from historic vicariance events and the sympatric distribution is the result of secondary contact. Revealing the pattern of divergence in these cryptic species complexes will provide valuable information for the mechanisms and processes of

speciation.

The discovery of cryptic species has important implications for conservation and economy. The kuruma shrimp *Penaeus japonicus* is a food species that is widely distributed in the Indo-West Pacific region. It is economically important in fisheries and aquaculture in Japan, Australia and many Southeast Asian countries (Rosenberry 2001) and is the first shrimp that was successfully cultivated with large scale farming established in Japan and other countries. Recent studies in population genetic structure of the kuruma shrimp in the Indo-West Pacific, however, revealed that this shrimp actually constitutes two genetically distinct varieties which probably represent two cryptic species based on mitochondrial DNA sequence, AFLP and microsatellite analyses (Tsoi et al. 2005, 2007). The two varieties are morphologically very similar and they only differ in the length of the banding pattern on the carapace (Tsoi et al. 2005, 2007). Variety I is found in Japan, Taiwan and China while variety II consists of populations from Southeast Asia, Australia and the Mediterranean. As the two species may differ in physiology and other biological characteristics, the discovery of cryptic species of kuruma shrimp has far-reaching implications in the fishery management and aquaculture practices of this important biological resource.

The implications of recognizing of cryptic species are even more important for group related to human health (e.g. *Anopheles* which transmits killing malaria; Lehr et al. 2005) and pest management (Wang et al. 1998). Thus, identifying cryptic species does more than just satisfy our curiosity of nature.

2.3 Molecular markers for delineating species, morphotypes and hybrids

Morphology is a phenotypic expression combining the effect of genetic composition and environmental factors. Different species can show similar adaptations to the same habitat leading to convergent/parallel evolution and morphologically indistinct cryptic species. On the other hand, genetically homogenous individuals can also develop different morphologies in response to different environmental selection pressure result in high level of intraspecific phenotypic variation. Both of these two alternatives will result in taxonomic confusion. We have gone through some examples of how molecular tools help us in settling the problem in the former. We will have a look on the latter in this section.

The intertidal rocky shore is a habitat with high interspecific competition and severe environmental stresses (e.g. heat, desiccation etc.). The lower limit of organisms' distribution on the shore is usually determined by biological factors like competition while the upper limit is restricted by the physical factors (Connell 1961). The trade-off between the physical and biological factors leads to the characteristic distribution pattern in intertidal organisms called "vertical zonation" (Stephenson & Stephenson 1972) and the zonation pattern plays an important role in the community structure and evolution in intertidal biota. *Tetraclita rufotincta* is a common barnacle species in the Red Sea. Achituv & Borut (1975) described morphological differences among individuals settled on different shore levels and allozyme analysis also revealed genetic differentiation among these individuals (Achituv & Mizrahi 1987). Thus individuals from three different shore levels were classified as three distinct

species, *T. achituvi*, *T. rufotincta* and *T. barnesorum* which occupy the lower, middle and upper shore levels respectively (Ross 1999). However, subsequent studies on mitochondrial 12S rRNA gene revealed only two genetically distinct species (Appelbaum et al. 2002). The authors used plastic plates to collect newly settled spats on different shore levels and carried out single strand conformation polymorphism analysis on the 12S rRNA gene on the collected spats. They found that settlers collected from lower shore levels belonged to the same lineage, presumably *T. achituvi*, while cryprids of the other lineage, *T. rufotincta*, mainly settled on mid- and higher shore. The specimens genetically assigned to *T. rufotincta* showed different phenotypes and this morphological plasticity enables the species to survive in wider range on the shore. The species status of *T. barnesorum* was demonstrated to be invalid and it only represents a phenotypic variant of *T. rufotincta*. Thus, early reports on allozyme differentiation between these species may be due to selection on alleles (Achituv & Mizrahi 1987). Similar cases were also reported in other species, for example, the rocky shore barnacle *Chthamalus anisopoma* develops different parietes shapes in response to predation pressure from the snail *Acanthina angelica* (Lively 1986, Mokady et al. 2000). The coral barnacle *Cantellius* shows a high degree of morphological variation in association with different host species, but with little genetic differentiation (Mokady et al. 1999). These studies revealed that the advantages of molecular methods in clarifying taxonomic status of morphologically diverse taxa and the knowledge of the basis of phenotypic variations allows us to examine the mechanisms of morphological innovation and pattern of speciation.

2.4 Molecular markers for identification of hybrids

Animal mitochondrial DNA (mtDNA) is an effective and widely adopted marker in phylogenetic and phylogeographic studies thanks to its high mutation rate, large copy number, haploid nature and the availability of universal primers (see Avise 2000). The maternally inherited mtDNA, however, cannot distinguish hybrid individuals from parental types. The phenotype of hybrids is presumably intermediate between the two parent species leading to taxonomic confusions and the case becomes even more complicated if hybrids are fertile. Thus, the analysis of the nuclear genome is desirable in addition to mtDNA markers. The development of amplified fragment length polymorphism (AFLP) by Vos and colleagues in 1995 provides an effective tool for whole genome array analysis for population genetic and fine scale taxonomic investigation. AFLP markers have the advantage of analyzing a large number of loci in the genome without the need for any prior knowledge on the genome. On the other hand, AFLP alleles are dominant, leading to difficulties in statistical analysis of the data. Other nuclear markers like sequences of variable non-coding regions including internal transcribed spacers (ITS) of nuclear rRNA and introns, or hypervariable microsatellite loci yield data sets that facilitate subsequent statistical analysis, but prior knowledge on the genome of the target organisms is required for primer design. Thus, the choice among these nuclear markers varies according the organisms under investigation as well as the question to be resolved. AFLP markers can be easily applied to non-model organisms with limited genetic information while microsatellites and introns would be more widely used in organisms with better knowledge on the genome like *Drosophila* and human.

McFadden and Hutchinson (2004) used ITS1 sequence to demonstrate that the soft coral species *Alcyonium hibernicum* is the hybrid of two closely related species, *A. coralloides* and *A.* sp. *M2*. On the other hand, Tsukaya et al. (2003) used nuclear ITS1 and alcohol dehydrogenase gene to investigate the taxonomic status of *Callicarpa* x *shirasawana*, an herb growing on the edge of sparse forests which has intermediate morphology to and is a putative hybrid of *C. japonica* and *C. mollis*. They found that the morphologically intermediate individuals are the progeny of hybrid backcrosses with *C. japonica*. The F1 hybrids are morphology similar to *C. mollis*. Sequences of nuclear loci were also applied to many other organisms including crabs (Imai & Takeda 2005), fishes (Redenbach & Taylor 2002; Carson & Dowling 2006), birds (Vallender et al. 2007) and plants (Isoda et al. 2000). These studies detected hybridization which would lead to confusion in morphological taxonomy.

The large number of loci generated from AFLP enables the accurate identification of hybrids using only small numbers of wild parents which is usually the case in threatened species. Congiu et al. (2001) applied AFLP to distinguish individuals in commercially targeted sturgeon species, *Acipenser naccarii* and *A. transmontanus*, revealing the potential for the AFLP technique in verifying hybrids providing valuable information for restocking and conservation projects for these threatened species. The multilocus nature of AFLP also allows the investigation on progeny after the first generation. For example, O'Hanlon et al. (1999) used AFLP to investigate the genetic structure of invasive thistles, *Onopordum* spp. in Australia. They found the occurrence of *O. acathium* and *O. illyricum* of European origin, as

well as a wide range of genetic intermediates of these two species in Australia. Moreover, they also discovered diagnostic fragments from other European species that were never recorded in Australia indicating a complex pattern of hybridization of these invasive species. Their study demonstrated the usefulness of AFLP in detecting subtle introgression. *Phylloscopus collybita collybita* and *P. c. brehmii* are two warblers found in Europe and the two subspecies are reported to have the potential to produce fertile hybrids (Helbig et al. 2001). Yet their study revealed very weak mitochondrial DNA introgression compared to a much higher level of nuclear gene flow shown by microsatellite loci (Helbig et al. 2001). AFLP analysis demonstrated a considerable number of individuals with intermediate genotypes existing among the "pure" parental species assigned by song and morphology. This result confirmed the male biased gene flow suggested by mtDNA and microsatellite data. The hybrids of heterogametic female are less viable in concordant with the prediction by Haldane's rule (Wu et al. 1996). Subsequent studies on other bird species (Secondi et al. 2006; Vallender et al. 2007) also demonstrated the advantages of AFLP in investigating the dynamic of hybridization..

2.4 Phylogeography of the marine biota through Pleistocene glaciations

Since the proposal of the term "phylogeography" by Avise et al. (1987), there is an increasing trend of applying various DNA markers in revealing the demographic responses of organisms to the glaciations and the role of geography in speciation driven by allopatric isolation in Pleistocene.

During the Pleistocene, the global climate fluctuated greatly following

oscillations between glacial and interglacial periods. In the marine environment, glacier expansion and lowering in sea level led to changes in temperature, habitats and ocean current pattern (Lambeck et al. 2002; Graham et al. 2003) that dramatically affected the distribution and abundance of organisms (Hewitt 2000; Jackson & Overback 2000). Species went extinct over large parts of their range during glacial maxima and only a small number of individuals survived in refugia. This contributes to genetic differentiation between different refugial populations and promoted speciation (Hewitt 1999, 2000; Dynesius & Jansson 2000). The species range expanded again from glacial refugia following regression of glacier and hybridization might occur at the narrow zones where the diverged genomes meet (Hewitt 2000, 2001, 2004).

In the northwestern Atlantic intertidal, there are several different scenarios among different organisms (Wares & Cunningham 2001; reviewed in Wares 2002). The glaciated habitats in the NW Atlantic are thought to be stressful for the rocky shore species and local extinction is expected. Rocky shore habitats were not found in the more temperate south. Thus, organisms would probably colonize from Europe where more hard substratum is available (van Oppen et al. 1995). Study on six intertidal taxa with amphi-Atlantic distribution found significantly higher genetic diversity in European populations than in Atlantic populations supporting long term persistence in Europe (Wares & Cunningham 2001). Most of the haplotypes found in Atlantic populations were shared with European populations in five of the six species studied (including the seastar *Asterias rubens*, the barnacle *Semibalanus balanoides*, the gastropod *Littorina obtusata* and *Nucella lapillus*, and the isopod *Idotea balthica*) indicating a postglacial colonization from Europe. However, evidence for the

persistence of survivors in the NW Atlantic during glaciation was also observed in *Semibalanus balanoides* as well as the mussel *Mytilus edulis* (Wares & Cunningham 2001). The authors suggested that the difference in survival ability among these studied organisms might be due to their life-history traits. The taxa with longer pelagic larval phase may have a higher chance to survive as it is easier for them to find the sparsely distributed substratum. However, the long term persistence in North Atlantic refugia were also be reported in many species regardless of their dispersal ability, such as the killifish *Fundulus heteroclitus* (Gonzalez-Villasenor & Powers 1990), the hermit crab *Pagurus longicarpus* (Young et al. 2002), the bivalves *Arctica islandica* (Dahlgren et al. 200), and the red alga *Chondrus crispus* (Chopin et al. 1996). The isolation through glaciation episodes led to genetic differentiation between populations from the north and south.

Similar pattern of genetic breaks between northern and southern populations arisen from allopatric glacial refugia is also observed in the northeastern Atlantic. A concordant genetic structuring is observed in various organisms including the barnacle *Balanus glandula* (Sotka et al. 2004), the intertidal fishes *Xiphister atropurpureus* (Hickerson & Cunningham 2005), the clingfish *Gobbiesox maeandricus* (Hickerson & Ross 2001), the sea cucumber *Cucumaria pseudocurata* (Arndt & Smith 1998) and gastropods (Kyle & Boulding 2000; Marko 2004). These studies indicated that the ecological differences between species may be more important than larval dispersal potential in determining their responses to climatic change (e.g., Kyle & Boulding 2000; Marko 2004). Although the possibility of other factors like oceanography, adaptation in shaping the observed genetic pattern could not be dismissed, the Pleistocene glaciation probably plays the dominant role in

generating the genetic differentiation while the other factors may help in maintaining, or even steeping the genetic cline. The increasing evidence of the presence of northern refugia changed the previous belief that most present northern biota are the result of colonization from the south during the postglacial period.

Yet the studies in NW Pacific are much more limited compared to the Atlantic and northeastern Pacific. A recent study on redlip mullet *Chelon haematochelius* revealed that populations in northwestern Pacific were isolated in three marginal seas, the Sea of Japan, East China Sea and the South China Sea due to lowered sea levels during the Pleistocene (Liu et al. 2007). Analysis of mitochondrial control region sequences identified three lineages. These lineages mainly correspond to the three marginal seas but overlapped partially in their distribution range. Mismatch distribution analysis, Fu's *F* test and Tajima's *D* test detected a strong signal of demographic expansion dated back to the late Pleistocene indicating a postglacial range expansion and secondary contact of these allopatrically derived lineages. Studies on several other fish species in the NW Pacific include Japanese sea bass (*Lateolabrax japonicus*), spotted sea bass (*Lateolabrax maculates*), Japanese anchovy (*Engraulis japonicus*) and Australian anchovy (*Engraulis australis*) (Liu 2006a. b) also revealed a population expansion following the retreat of glacier and genetic structuring was observed in some of these species as a result of refugial isolation. Yet our understanding on the responses of other biota to glaciation is far from complete. Further studies on a wider array of organisms combined with information from ecology, physiology and oceanography will be needed to elucidate the mechanisms governing the alternatives between persistence and re-colonization in different organisms.

Chapter 3

Cryptic Diversity of the Acorn Barnacle, *Tetraclita squamosa* Species Complex in East Asia

3.1 Introduction

The acorn barnacle *Tetraclita* is a common space occupier in the intertidal zone of tropical and sub-tropical waters (Newman & Ross 1976) and can play an important role in the ecology of intertidal assemblages (see Reimer 1976a, b; Bertness et al. 1998). The variable morphology of *Tetraclita* has resulted in taxonomic confusion. Presently, at least 20 *Tetraclita* species have been identified (Ross 1969, 1999; Newman & Ross 1976). *Tetraclita squamosa* was first described by Bruguière (1789) and re-classified by Darwin (1854) into 12 subspecies based on variation in parietes color and opercular plate and soft body morphologies. In East Asia, the three subspecies, *T. squamosa squamosa*, *T. s. japonica* and *T. s. formosana* have been recently separated as distinct species (*T. squamosa, T. japonica* and *T. formosana*) based on morphological and molecular analyses (Yamaguchi 1987; Hasegawa et al. 1996; Chan 2001, 2003). *Tetraclita squamosa* has green parietes (Fig 1.1B) and has the widest distribution throughout the Indo-Pacific region (Newman & Ross 1976; Ren & Liu 1979).

Comparing the previous morphological descriptions of *Tetraclita squamosa* from different geographical regions in East Asia reveals variation in morphology and zonation patterns, suggesting that a species complex may still exist in '*Tetraclita squamosa*' from different regions. Yamaguchi (1987), for example, believed there

were no differences in cirral morphology between *T. squamosa* and *T. japonica* in Japan, whilst Ren & Liu (1979) and Chan (2001) reported that the third cirrus of *T. japonica* had cuspidate setae, that were absent in *T. squamosa*. Pilsbry (1916) reported that the tergum of *T. squamosa* from Japan, as illustrated by Krüger (1911) was different from that of *T. squamosa* collected from China and the Philippines. The vertical zonation of *Tetraclita squamosa* also exhibits geographical variation. In Japan, *T. squamosa* occurs sympatrically with *T. japonica* and *T. formosana* in the mid shore (Yamaguchi 1987), whilst in southern China, *T. squamosa* is a low shore inhabitant, separated from *T. japonica* which is found on the mid shore (Ren & Liu 1979; Chan 2001). Whether these morphological and ecological differences in *T. squamosa* are induced by environmental factors or actually represent distinct genetic difference is obscure. There have been no genetic studies on the population differentiation of this species in East Asia that would indicate whether such differentiation, if exists, suggests reproductive isolation or a gradual cline of variation. Such information is crucial for an evaluation of the taxonomic status of *T. squamosa* that is important for further studies on the biogeography, phylogeny and ecology of *Tetraclita*.

In the present study, DNA sequences of the mitochondrial 12S rRNA, cytochrome *c* oxidase I (COI) and first internal transcribed spacer (ITS1) of nuclear rRNA of *Tetraclita squamosa* from Honshu, Japan to Hong Kong were determined to estimate the genetic divergence of *Tetraclita squamosa* in East Asia. The three genes have commonly been used in delimiting species of arthropods (e.g., Stewart et al. 2004; Tsoi et al. 2005; Tixier et al. 2006). *Tetraclita japonica* and *T. formosana* were also studied for comparison.

Data presented in this Chapter have been published in Chan et al. (2007a).

3.2 Materials and Methods

3.2.1 Sample collection

Tetraclita squamosa were collected from tidal levels where they are abundant in Shimoda and Kurosaki of Japan, Okinawa, Badoutz of Taiwan, Xiamen and Hong Kong (Fig. 3.1). *T. formosana* were collected from Badoutz, Taiwan, while *T. japonica* were collected from Hong Kong. Study sites in Honshu, Okinawa and Taiwan are located in northwestern Pacific waters, whilst Hong Kong and Xiamen are located in southern China waters. The barnacles were preserved in 95% ethanol prior to laboratory analysis. The samples were collected from 2003 to 2006 through the collaboration with Dr BKK Chan (Academia Sinica) and the assistance of several colleagues (see acknowledgements).

3.2.2 DNA extraction, PCR and sequencing

Total genomic DNA was extracted from whole soft tissue of individual barnacles using the QIAamp Tissue Kit (Qiagen). Partial sequences of mitochondrial 12S rRNA and COI were amplified using the universal primers 12Sai and 12Sbi (Simon et al.. 1994), and LCO1490 and HCO2198 (Folmer et al. 1994), respectively. The complete ITS1 region was amplified using SP-1-5'138 and SP-1-3' (Chu et al. 2001). The amplification of the three genes was conducted in a reaction mix containing 1 μL of template DNA, 1X PCR reaction buffer, 2 mM $MgCl_2$, 200 nM of

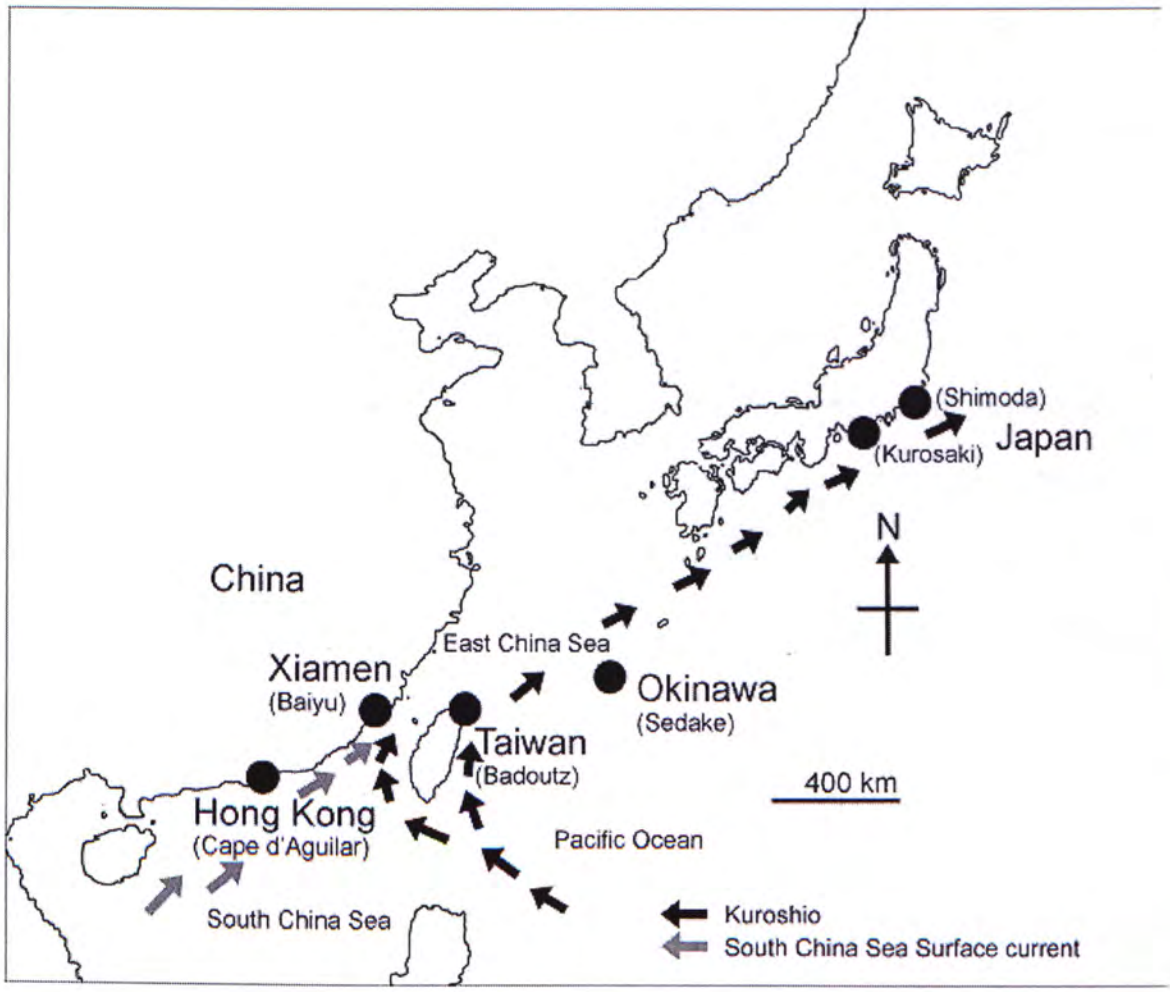


Fig. 3.1 Sampling locations for *Tetraclita.* Study sites in the Honshu, Japan included Shimoda at the Izu Peninsula and Kurosaki. Sampling was conducted at Sedake in Okinawa, Badoutz in Taiwan, Baiyu in Xiamen and Cape d'Aguilar in Hong Kong. The directions of the Kusoshio and South China Sea Surface Current during summer (reproductive season of *Tetraclita*) were illustrated by black and grey arrows respectively.

each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profile for 12S rRNA was as follows: 3 min at 94°C for initial denaturation, 33 cycles of 30 s at 94°C, 30 s at 50°C, 40 s at 72°C and a final extension for 3 min at 72°C. A similar profile was employed for COI and ITS1 with an annealing temperature of 47°C and 54°C, respectively. The PCR products were then purified using QIAquick gel purification kit (Qiagen). Sequences were generated using the same sets of primers and determined on an Applied Biosystems 3100 automated sequencer using the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol. Sequences were determined from specimens collected from all localities except Kurosaki, Japan.

3.2.3 Sequence analysis

Sequences were aligned using CLUSTAL W (Thompson et al. 1994) with default gap weighting parameters, and adjusted by eye. The alignment of COI sequences was confirmed by translating the aligned DNA sequences into amino acid sequences. A matrix of genetic distance was generated using Kimura's two-parameter method (Kimura 1980) with MEGA v3.1 (Kumar et al. 2004). Both missing data and gaps were treated as missing data. The sequence data sets of the three genes were analyzed independently to determine phylogenetic relationships between individuals of *Tetraclita squamosa*, *T. japonica* and *T. formosana*. Neighbour-joining (NJ) analysis was performed using PAUP* v4.0 b10 (Swofford 2000). Kimura 2-parameter distance was used with 1000 replicates used for bootstrapping.

3.3 Results

The number of sequences determined for each of the three genes is shown in Table 3.1. All sequences were deposited with GenBank (Accession nos. DQ363680-DQ363748). The aligned COI fragments consisted of 628 bp of which 159 were variable. The aligned 12S rRNA sequences included 309 bp, with 33 variable sites. Length polymorphisms in ITS1 was observed between individuals from NW Pacific (Japan, Okinawa and Taiwan) and S China (Xiamen and Hong Kong) (258 bp and 249 bp, respectively), but not among the localities in each of the regions. The ITS length in *Tetraclita japonica* and *T. formosana* was 258 bp. The aligned data set contained 262 sites, of which 56 were variable. Sequence ambiguities (i.e. double peaks in the chromatograms) were found in some ITS sequences that might be due to presence of multiple copies in the individuals from which the sequences were derived. In most cases, ambiguities were found only at one site, yet in a few individuals of *T. squamosa* from Taiwan and Okinawa, the number of ambiguities amounted up to 9. These sites were treated as missing data in NJ analysis.

All three gene trees based on NJ analysis (Figs. 3.2, 3.3, 3.4) showed similar topology. Individuals of *T. squamosa* were consistently divided into two distinct clades with high bootstrap support (>90%). The first clade consisted of all *T. squamosa* from Hong Kong and Xiamen, whereas the second one consisted of all *T. squamosa* from Japan, Okinawa and Taiwan. They were more closely related to each other than to either *T. japonica* or *T. formosana,* which clustered as the third group.

Table 3.1 Pairwise comparison of genetic distance based on Kimura's two-parameter method generated from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene (628 bp), 12S rRNA (309 bp) and the first internal transcribed spacer (ITS) region 1 (262 bp) between different populations of *T. squamosa* (TW: Taiwan; OK: Okinawa; JP: Japan; XM: Xiamen; HK: Hong Kong), *T. japonica* (TJ) and *T. formosana* (TF). Data represent range with mean values in parentheses.

Population	TW	OK	JP	XM	HK	TJ	TF
COI sample size (n)	7	7	5	8	8	2	2
TW	0-0.013 (0.007)						
OK	0.002-0.016 (0.009)	0.003-0.015 (0.012)					
JP	0.005-0.015 (0.010)	0.006-0.019 (0.012)	0.008-0.018 (0.013)				
XM	0.147-0.161 (0.154)	0.147-0.163 (0.157)	0.149-0.166 (0.157)	0-0.010 (0.006)			
HK	0.147-0.157 (0.152)	0.147-0.161 (0.156)	0.149-0.166 (0.155)	0.003-0.010 (0.004)	0-0.003 (0.002)		
TJ	0.209-0.214 (0.212)	0.207-0.218 (0.211)	0.203-0.214 (0.209)	0.188-0.209 (0.198)	0.192-0.205 (0.199)	0.016	
TF	0.214-0.221 (0.218)	0.214-0.225 (0.217)	0.209-0.221 (0.215)	0.192-0.207 (0.199)	0.196-0.203 (0.200)	0.011-0.016 (0.14)	0.011
12S sample size (n)	4	3	4	3	5	2	2
TW	0-0.007 (0.003)						
OK	0-0.007 (0.002)	0-0.003 (0.002)					
JP	0-0.010 (0.003)	0-0.010 (0.003)	0-0.007 (0.003)				
XM	0.037-0.043 (0.041)	0.037-0.043 (0.040)	0.037-0.040 (0.039)	0.003-0.007 (0.004)			
HK	0.037-0.040 (0.038)	0.037-0.040 (0.038)	0.037	0-0.003 (0.002)	0		
TJ	0.082-0.090 (0.086)	0.082-0.086 (0.085)	0.086-0.094 (0.088)	0.086-0.090 (0.089)	0.086	0.003	
TF	0.079-0.086 (0.082)	0.079-0.082 (0.081)	0.082-0.090 (0.084)	0.082-0.086 (0.085)	0.082	0.003-0.007 (0.005)	0
ITS1 sample size (n)	7	7	4	8	8	2	2
TW	0-0.025 (0.009)						
OK	0-0.033 (0.008)	0-0.033 (0.006)					
JP	0-0.033 (0.010)	0-0.033 (0.009)	0-0.033 (0.016)				
XM	0.070-0.096 (0.080)	0.070-0.096 (0.077)	0.077-0.096 (0.083)	0-0.004 (0.001)			
HK	0.070-0.096 (0.079)	0.070-0.096 (0.077)	0.077-0.096 (0.082)	0-0.004 (0.001)	0-0.004 (0.001)		
TJ	0.163-0.187 (0.174)	0.167-0.187 (0.176)	0.175-0.187 (0.181)	0.192-0.198 (0.195)	0.192-0.198 (0.195)	0	
TF	0.163-0.187 (0.174)	0.167-0.187 (0.176)	0.175-0.187 (0.181)	0.192-0.198 (0.195)	0.192-0.198 (0.195)	0	0

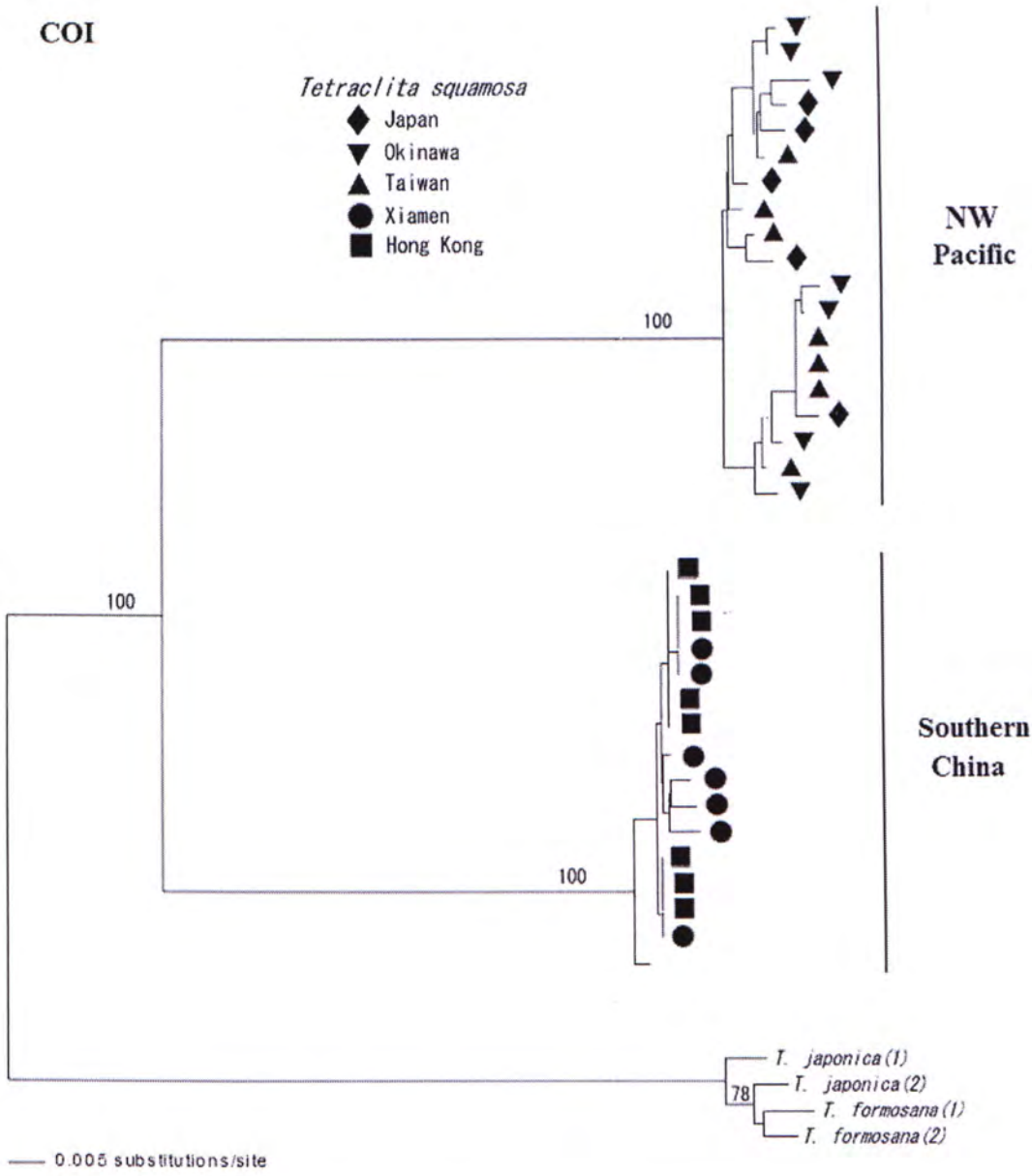


Fig. 3.2 Neighbour-joining (NJ) tree of five *T. squamosa* populations and two outgroup taxa, *T. japonica* and *T. formosana* based on COI. The percentages of bootstrap replicates are shown above the branch for all values >75%. Individuals from each population are represented by different symbols. The numbers in parentheses for *T. japonica* and *T. formosana* denote the two different individuals analyzed.

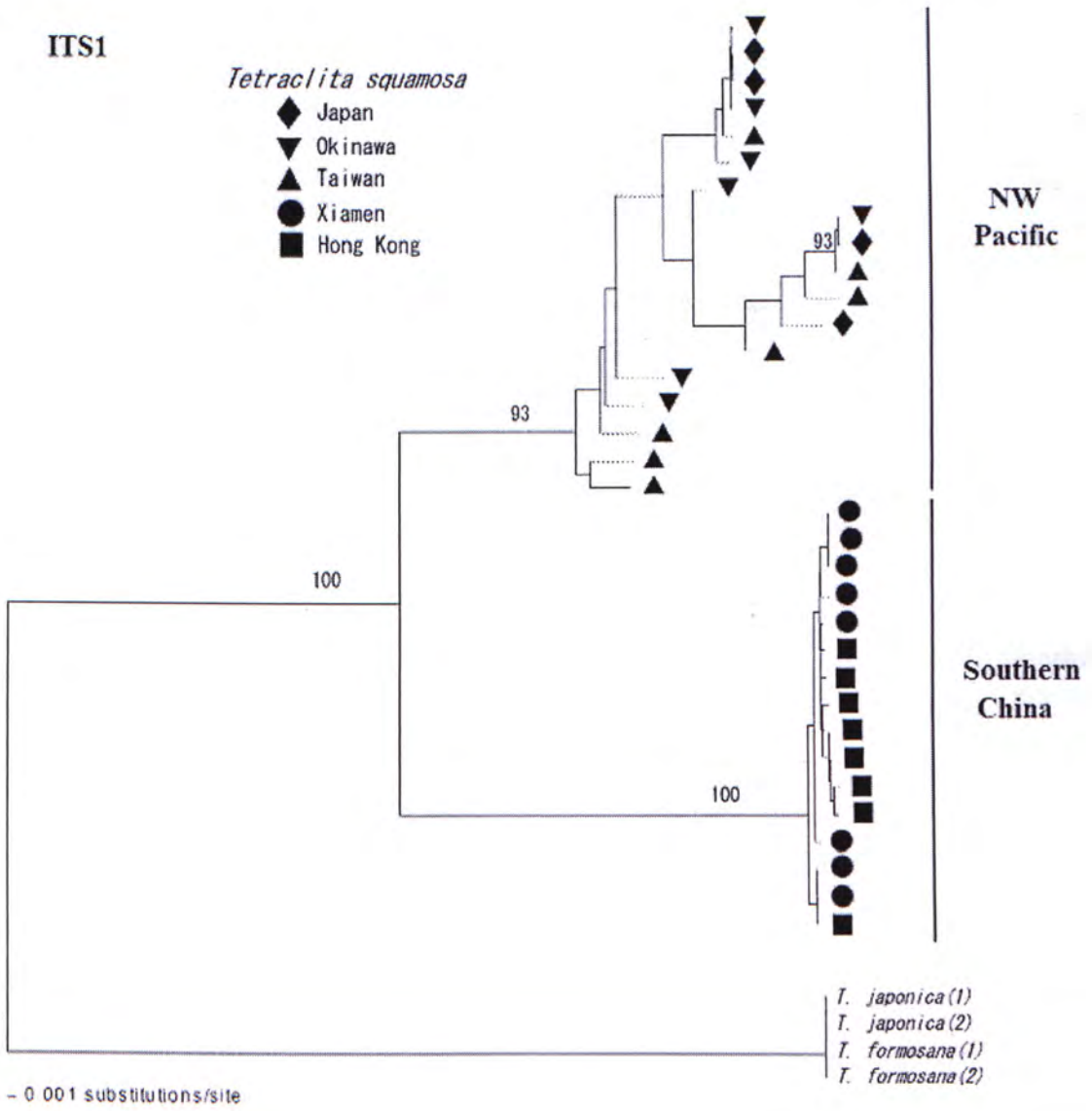


Fig. 3.3 Neighbour-joining (NJ) tree of five *T. squamosa* populations and two outgroup taxa, *T. japonica* and *T. formosana* based on ITS1. The percentages of bootstrap replicates are shown above the branch for all values >75%. Individuals from each population are represented by different symbols. The numbers in parentheses for *T. japonica* and *T. formosana* denote the two different individuals analyzed.

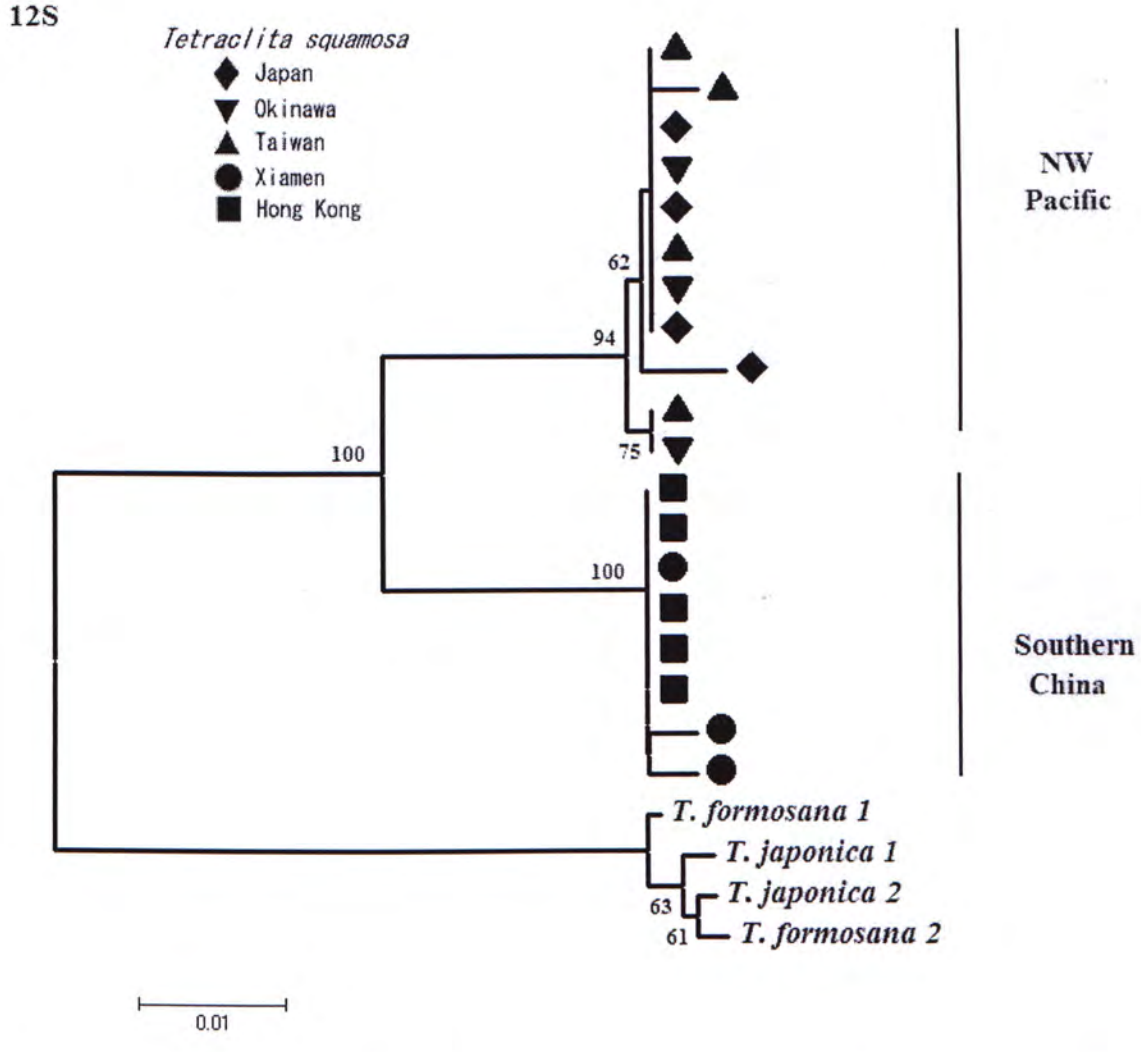


Fig. 3.4 Neighbour-joining (NJ) tree of five *T. squamosa* populations and two outgroup taxa, *T. japonica* and *T. formosana* based on 12S rRNA. The percentages of bootstrap replicates are shown above the branch for all values >75%. Individuals from each population are represented by different symbols. The numbers in parentheses for *T. japonica* and *T. formosana* denote the two different individuals analyzed.

Among the three markers analyzed, COI showed the highest sequence divergence (Table 3.1). Even in this most variable marker, the value of sequence divergence within each clade of *T. squamosa* was very low, less than 1.5% (Table 3.1). In each clade, individuals from the same locality did not group together. The two forms differentiated by more than 15%, 3.5% and 7% in COI, 12S rRNA and ITS1, respectively. These values were much higher than those within the clades, showing that the two were genetically distinct. On the other hand, the COI and 12S rRNA of *T. formosana* and *T. japonica* only differed by <1.6% and <1%, respectively. Their ITS sequences were identical, indicating that the genetic differentiation between these two species was minimal. The sequence divergence between the cluster of these two species and the two *T. squamosa* forms was higher than 19%, 7.5% and 16% in COI, 12S rRNA and ITS1, respectively.

3.4 Discussion

3.4.1 Genetic variations of Tetraclita squamosa

In the past, *Tetraclita squamosa* has been regarded as a complex of subspecies showing great morphological variations, resulting in taxonomic confusion (Darwin 1854; Pilsbry 1916). Recently, on the basis of detailed morphological and molecular analyses, a number of subspecies have been raised to species level (Hasegawa et al.. 1996). In the Red Sea, for example, *Tetraclita squamosa rufotincta* was re-classified into *T. rufotincta*, *T. barnesorum* and *T. achituvi* (Ross 1999) based on differences in morphology and vertical distribution between species (also see genetic analysis in Achituv & Mizrahi 1987). In the present study, *T. squamosa* from

the NW Pacific and south China exhibit significant genetic differentiation (hereafter named as NW Pacific form and S China form respectively) indicating that the reported differences in zonation patterns and morphology reflect genetic difference rather than geographical variations.

Hasegawa et al. (1996) reported the 3' sequences of COI of *T. squamosa* in Japan, which overlap with the 5' sequences we determined by 273 bp. Comparing this region shows that our COI sequence for the NW Pacific form differs from the *T. squamosa* sequence of Hasegawa et al. (1996) only by <1.5%. Yet the two forms distinguished in our study differ by 15-16% in COI divergence, which is comparable to values of other congeneric species of barnacles, e.g., ~15% in *Chthamalus*, and higher than 9.3% in *Euraphia* (Wares 2001). This supports the morphological pattern that the two forms of *T. squamosa* represent two genetically differentiated species. The divergence of 12S rRNA (~3.5-4.2%) between the two forms is slightly lower than values between other congeneric species of barnacles that have been reported. The interspecific 12S rRNA sequence divergence of the coral-inhabiting *Cantellius* and *Savignium* are 4.1-5.0% and 9.2-13.9% respectively (Mokady et al. 1999), whereas rock-inhabiting *Chthamalus* showed much higher (~15-18%) interspecific sequence divergence (Mokady et al. 2000). However, the nearly identical 12S rRNA sequences within each form of *T. squamosa* and the substantial divergence between the two forms do support them as two genetically distinct taxa. The comparatively low sequence divergence in the 12S rRNA gene may indicate that the two forms represent recently evolving sister taxa.

Sequence divergence alone should not be used to decide taxonomic ranks

and the maternally inherited mitochondrial DNA cannot distinguish hybrid individuals between taxa. To elucidate whether the two *T. squamosa* forms are reproductively isolated, we also determined the nuclear ITS1 region, which has been shown to be effective in determining interspecific hybrids in many organisms (e.g., McFadden & Hutchinson 2004; Imai & Takeda 2005). From our results, there are no ITS genotypes shared between the two *T. squamosa* forms, which show length polymorphism and significant sequence divergence (~8%). These results provide no evidence for interbreeding between the two forms of *T. squamosa.* On the other hand, the ITS sequences of *T. japonica* and *T. formosana* are identical and the sequence divergence in the two mitochondrial genes are very low. Hasegawa et al. (1996) also reported that the 3' region of COI differs only by 1.2% between the two species. The genetic differentiation between of these two species would be further explored in subsequent sections (see Chapters 4 and 5).

Apart from genetic differentiation, a parallel study by Dr B.K.K. Chan (Academia Sinica) based on scanning electron microscope analysis of the cirri structure and morphometric analysis on the opercular plate of the same individuals used in the present study also revealed further morphological difference between the two forms. The S China form of *T. squamosa* has a beaked tergum, a sharper tergal spur and a longer articular ridge-basal margin distance in the scutum when compared to the NW Pacific form (Chan et al. 2007a). The patterns of the tergo-scutal flaps and set al. types of the cirrus I in the two forms also exhibit diagnostic differences (Chan et al. 2007a). They also differ in zonation patterns. S China form is a low shore inhabitant, forming its own belt below the *T. japonica* zone on the mid shore (Ren & Liu 1979; Chen et al. 1987; Chan 2001, Chan et al. 2001). In contrast, NW Pacific

form co-exists with *T. japonica* and *T. formosana* in the mid shore in the Pacific waters of Japan (see Yamaguchi 1987).

3.4.2 Identification of new species

All of these morphological, ecological and molecular evidences suggest there are two distinct species in the previously described *Tetraclita squamosa* in East Asia. From the descriptions of the type *Tetraclita squamosa* (= *Balanus squamosa* (Bruguière 1789; Gmelin 1789)) and the subspecies *Tetraclita porosa* var *viridis* (Darwin 1854), there is no information on the opercular plate morphology. The type locality in Bruguière 1789 is from Tranquebar, India, which is not located in East Asia. It is, therefore, not possible to assign which geographic form represents *Tetraclita squamosa* based on the earliest type descriptions. Pilsbry (1916) assumed that the type specimens of Bruguière (1789) and Gmelin (1789) were collected from China or the Philippines, based on their morphological descriptions. Pilsbry (1916) renamed the subspecies, *Tetraclita squamosa squamosa* as synonymous with *Tetraclita porosa viridis* Darwin 1854, based on samples from Hong Kong, China and the Philippines. From the sampling locations in Pilsbry (1916), the present study refers the south China form as *Tetraclita squamosa* and identifies the NW Pacific form as a new species. *Tetraclita squamosa* is the only species described so far in the genus *Tetraclita* that has green parietes (Darwin 1854; Pilsbry 1916; Ren & Liu 1979). In the present study, both species identified from *Tetraclita squamosa* have green parietes. The new species does not represent any other previously described *Tetraclita* species in East Asia. The NW Pacific form has thus been described as a new species, namely *Tetraclita pacifica* (Chan et al. 2007a). However, it was

subsequently found that this name is preoccupied by Pilsbry 1928's *Tetraclita wireni pacifica*. *Tetraclita wireni* is now assigned to another genus *Tesseropora* (see Newman & Ross, 1976). As *Tetraclita pacifica* is a junior homonym of *Tetraclita wireni pacifica*, the new name *Tetraclita kuroshioensis* is given to the new species (see Appendix A in Chan et al. 2007b).

3.4.3 Biogeography and distribution patterns

Tetraclita kuroshioensis and *T. squamosa* have different geographic distributions. *Tetraclita kuroshioensis* is distributed on the Pacific coast of Japan, Okinawa and Taiwan and further to the Oceania region. On the other hand, *T. squamosa* is common along the south China coastline in the Taiwan Strait and South China Sea. Difference in geographical distribution between *Tetraclita kuroshioensis* and *T. squamosa* can be affected by ocean currents and larval supply patterns. The Pacific coast of Japan, Okinawa and Taiwan are affected by the Kuroshio (Ishizaka et al. 1992; Ito et al.. 1995; Chiang et al. 1997; Lee & Chao 2003) flowing northwards from Luzon Strait. Xiamen is located in the Taiwan Strait where the ocean currents are influenced by seasonal monsoons. In summer, the waters in the Taiwan Strait are mainly from the South China Sea Current and the Kuroshio (spring) which enters the strait from the Penghu Channel (Jan et al. 2002). In late summer to winter, the water in the strait is influenced by the China Coastal Current, which flows southward under the effect of northwest monsoons. The entry of the South China Sea Current and the Kuroshio is blocked at the Penghu Channel during the winter months (Jan et al. 2002; Fig. 3.1). In Hong Kong, the waters in summer are affected by South China

Sea Current whilst in winter, both the Kuroshio and the China Coastal Current from the Taiwan Strait enter Hong Kong (Morton et al. 1996). *Tetraclita* reproduces in the summer due to elevated water temperature (Cai & Huang 1986; Chan & Williams 2004) and settlement occurs in late summer, from August to October (Chen et al. 1987; Chan & Williams 2003, 2004), suggesting that the geographical distribution is affected by the ocean currents in summer. *Tetraclita kuroshioensis* is distributed in the NW Pacific and Oceania, and its larval pool is probably associated with the Kuroshio. *Tetraclita squamosa* have planktotrophic larvae which complete development in 14 days (Chan 2003). Larvae of *Tetraclita squamosa* may not, therefore, disperse throughout the Indo-Pacific region and may probably associate with the South China Sea Currents (Fig. 3.1). This restricts the occurrence of *T. squamosa* in the South China Sea and the Chinese coastline in the Taiwan Strait.

Tetraclita squamosa is the species in the genus *Tetraclita* reported to have the widest geographical distribution, including the Indo-Malayan-Pacific waters. In the present study, two distinct species are identified from *Tetraclita squamosa* in East Asia. A recent study in our laboratory found that "*T. squamosa*" from Singapore is genetically and morphologically differentiated from both *T. squamosa* and *T. kuroshioensis* studied here and the Singapore samples were described as another new species, *Tetraclita singaporensis* (Chan et al. 2007b). Moreover, comparing the 12S rRNA sequence of *T. squamosa* from Australia (Pérez-Losada et al. 2004) and "*T. squamosa*" from the present study shows a divergence >10%, indicating that '*T. squamosa*' in Australia may represent yet another, unidentified, cryptic species. It appears that there may still be more species in the *Tetraclita squamosa* complex. Further studies are needed to confirm the identity and geographical distribution of

Tetraclita squamosa in the South China Sea (including Vietnam, Malaysia and Indonesia), Australia and the Indian Ocean.

Chapter 4

Lack of genetic differentiation among acorn barnacles *Tetraclita japonica* and *Tetraclita formosana* populations in mtDNA COI, control region and nuclear ITS1 sequences

4.1 Introduction

In East Asia, *Tetraclita japonica* and *Tetraclita formosana* have been classified as distinct species separated from the subspecies complex of *T. squamosa* based on allozyme and DNA analyses (Yamaguchi, 1987; Hasegawa et al., 1996). The two taxa are morphologically similar and the diagnostic difference is that *T. japonica* has grey or purplish-grey parietes whilst parietes color in *T. formosana* is pink (Hiro, 1939) (Fig. 1.1A). They also differ in geographical distribution. In Japan, both *T. japonica* and *T. formosana* are reported, whereas in Taiwan, *T. formosana* exists in high abundance and almost no *T. japonica* is found (Hiro, 1939; Yamaguchi, 1987).

However, previous studies only based on limited number of samples and the genetic differentiation revealed is minimal. In the study by Hasegawa et al. (1996), the COI sequence divergence between *T. japonica* and *T. formosana* was found to be 1.2% which is even smaller than intra-specific divergence reported in many other barnacles species (e.g. ~2% in *Balanus glandula* (Sotka et al.. 2004) and *Chthamalus proteus* (Zardus and Hadfield 2005)). Recent studies on a few individuals of the two taxa indicate little genetic differentiation in both nuclear (ITS1) and mtDNA (12S rDNA and COI) (see Chapter 3). *Tetraclita japonica* and *T. formosana* could,

therefore, be two color morphotypes of the same species with different geographical distributions and under divergent selection pressure. The present study investigated the mitochondrial COI and control region, and nuclear ITS1 sequences of *T. japonica* and *T. formosana* using samples over their geographical range to evaluate their taxonomic status and explore the evolutionary basis of differences in parietes color and geographical distribution. Parts of the data presented in this Chapter have been published in Tsang et al. (2007).

4.2 Materials and Methods

4.2.1 Samples collection

Samples of *Tetraclita* were collected from Honshu, Japan (Kominato, Kurosaki and Wakayama), Okinawa (Sedake), Taiwan (Badoutz, Three Fairy Platforms, and Kenting), Xiamen (Baiyu), and Hong Kong (Cape d'Aguilar) (Fig. 4.1; Table 4.1). In Honshu, *T. japonica* was the most common species and it occurs in two color forms (grey and purplish-grey) whilst *T. formosana* had low abundance (~ < 5% cover on shores). In Okinawa, *T. formosana* existed in low abundance and no *Tetraclita japonica* was found. In Taiwan, *Tetraclita formosana* was common whilst *T. japonica* existed in low abundance. Thus only three individuals each could be collected from Sedake, Okinawa and Yeliu, Taiwan in this study. In Hong Kong and Xiamen, *T. formosana* was absent and *T. japonica* was common on the mid shores. All the samples were stored in 95% ethanol prior to laboratory analysis. The samples were collected from 2003 to 2006 through the collaboration with Dr BKK Chan (Academia Sinica) and the assistance of several colleagues (see acknowledgements).

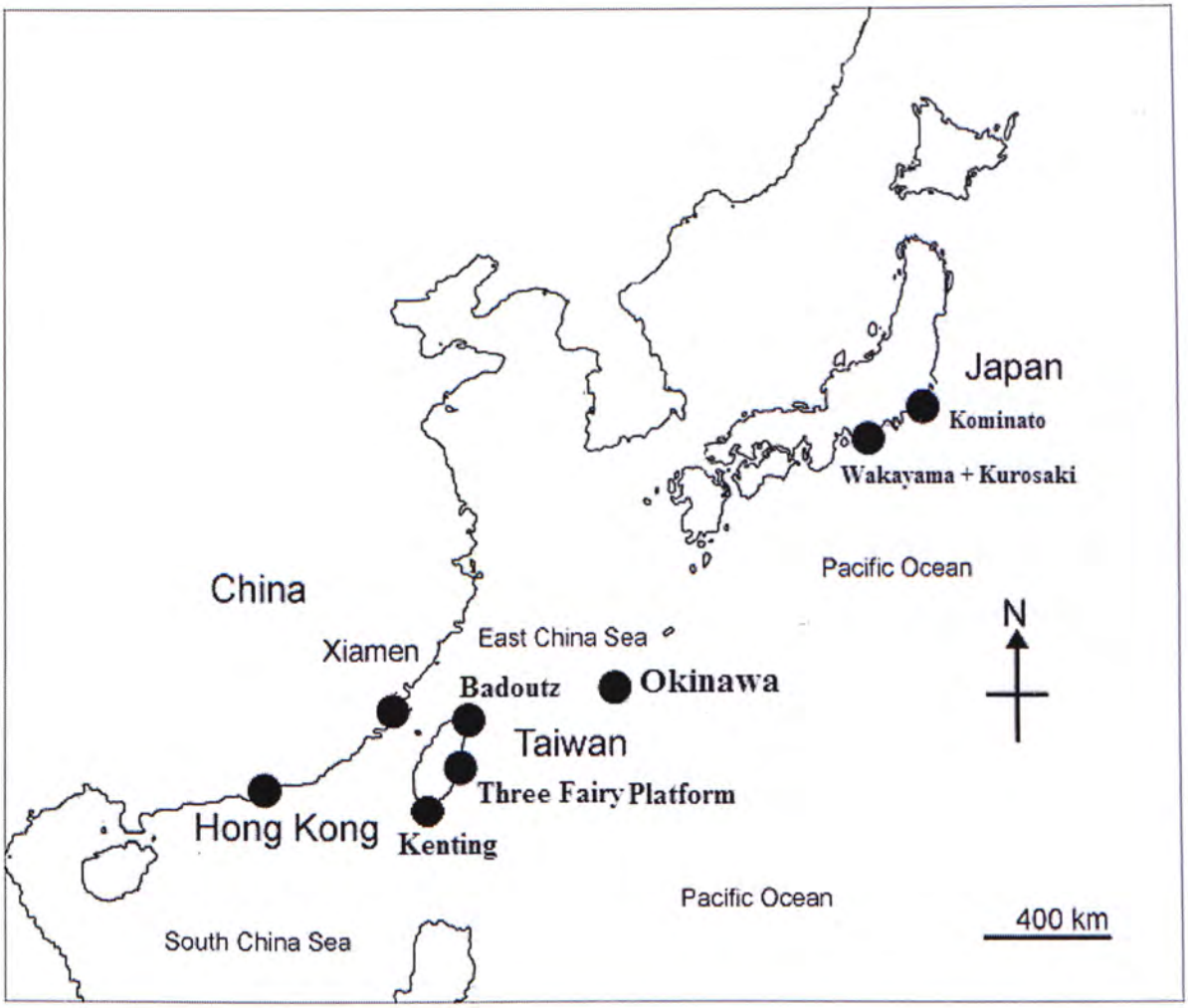


Fig 4.1 Sampling locations in SE Asia for *Tetraclita japonica* and *Tetraclita formosana*. *Tetraclita japonica* were collected from Kominato, Shirahama in Honshu, Japan, Badoutz in Taiwan, Baiyu in Xiamen and Cape d'Aguilar in Hong Kong. *T. formosana* was collected from Kurosaki and Wakayama in Honshu, Japan, Sedake in Okinawa, Badoutz in N. Taiwan and Three Fairly Platforms in E. Taiwan and Kenting in S Taiwan.

4.2.2 DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from whole soft tissue of individual barnacles using the commercial QIAamp Tissue Kit (QIAGEN). Partial sequences of mitochondrial COI were amplified using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994). For amplifying the control region, we designed the primer, MetTC: 5'-TGATGAATGTGCGCACCTCTAC-3', based on the conserved sequence in the methionine tRNA flanking the control region of the complete mitochondrial DNA sequence of *T. japonica* (GenBank accession no. AB126701). The complete control region sequences were amplified using MetTC and the universal primer 12Sbi (Simon et al. 1994). The complete ITS1 region was amplified using SP-1-5'138 and SP-1-3' (Chu et al. 2001). The amplifications were conducted in a reaction mix containing 1 μL of template DNA, 1X PCR reaction buffer, 2 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profile for COI and ITS1 was as follows: 3 min at 94°C for initial denaturation, then 33 cycles of 30 s at 94°C, 30 s at 50°C, 40 s at 72°C with a final extension for 3 min at 72°C. A similar profile was employed for the control region with an annealing temperature 60°C and an extension time of 1.5 min in each cycle. The PCR products were then purified using the QIAquick gel purification kit (QIAGEN) according to manufacturer's instructions. Sequences were generated using the same sets of primers and an Applied Biosystems (ABI) 3100 automated sequencer using the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol.

4.2.3 Sequence analysis

Sequences were aligned using CLUSTAL W (Thompson et al. 1994) with default gap weighting parameters, adjusted by eye. Alignment of COI sequences was confirmed by translating the aligned DNA sequences into amino acid sequences. A matrix of genetic distance was generated using Kimura's two-parameter method (Kimura 1980) with MEGA v3.1 (Kumar et al. 2004). Both missing data and gaps were treated as missing data. The sequence data sets of the two genes were analyzed independently to determine the phylogenetic relationships between individuals of *T. japonica* and *T. formosana*. Neighbour-joining (NJ) analysis was performed using PAUP* v4.0 b10 (Swofford 2000). Kimura 2-parameter (K2P) distance was used and with 1 000 bootstrap replicates. Sequences from the closely related species, *T. squamosa* and the newly discovered *T. kuroshioensis* were used as outgroups. A haplotype network was constructed using the 95% parsimony criterion as implemented by the program TCS version 1.13 (Clement et al. 2000) to determine the genealogical relationship among haplotypes.

Genetic differentiation between the two species and among geographical regions was examined using analysis of molecular variance (AMOVA, Excoffier et al. 1992) as implemented in ARLEQUIN version 3.0 (Excoffier et al. 2005) with K2P distance and 1000 random permutations to test for statistical significance. All individuals collected from the same site were treated as a single population. The analysis was first carried out to detect any genetic differentiations between two color forms of *T. japonica* (grey or purplish-grey) from Japan. Then AMOVA was performed for three hierarchical groupings of the data. The first level compared

variations among individuals within each population. The second level examined genetic structure among populations of each taxon. Finally, variations between *T. japonica* and *T. formosana* were studied by combining all geographical samples. This analysis provided insight into the proportion of genetic variations attributable to within-population (Φ_{ST}), within-group (Φ_{SC}), and among-group (Φ_{CT}) differences.

4.3 Results

4.3.1 Genetic divergence

The number of sequences determined for each of the three genes is shown in Table 4.1. All sequences were deposited to GenBank (Accession nos. DQ645827-DQ645887, DQ647704-DQ647770, DQ363748, DQ645827-DQ645887, EF051634-EF051698). The aligned COI fragments consisted of 657 bp of which 38 of 78 polymorphic sites were parsimony informative. The aligned control region (CR) sequences included 265 bp (individual sequence length ranging from 260-263 bp), with 112 variable sites of which 72 were parsimony informative. The aligned ITS1 sequences including those from outgroup taxa consisted of 262 sites. Of the 54 variable sites, 49 were parsimony informative. Only four genotypes were identified in the ITS1 sequences from a total of 38 individuals of *T. japonica* and *T. formosana*, and 35 individuals shared the same genotype. Each of the other three genotypes occurred only in a single individual and they differed in one or two bases. They differed from *T. squamosa* and *T. kuroshioensis* by ~20% and ~17% respectively.

Sequence divergences of two mitochondrial genes within either *T. japonica* or *T.*

Table 4.1 Collection localities (from south to north for each taxon), abbreviations and numbers of ITS1, COI and control region sequences determined.

	Population	Abbreviation	Number of ITS1 sequences obtained	Number of COI sequence obtained	Number of control region sequences obtained
T. japonica	Hong Kong, China	J-HK	4	11	16
	Xiamen, China	J-XM	3	6	5
	Badoutz, Taiwan	J-BA	3	4	3
	Wakayama, Honshu, Japan	J-WA	4	3	17
	Kominato, Honshu, Japan	J-KO	4	15	14
	Total		18	39	55
T. formosana	Kenting, Taiwan	F-KT	2	-	18
	Three Fairy Platforms, Taiwan	F-TF	3	4	10
	Badoutz, Taiwan	F-BA	5	14	14
	Okinawa, Japan	F-OK	4	3	8
	Kurosaki, Honshu, Japan	F-KU	2	3	3
	Wakayama, Honshu, Japan	F-WA	4	2	12
	Total		20	28	65
T. squamosa	Hong Kong, China		2	2	2
T. kuroshiensis	Badoutz, Taiwan		2	2	2

formosana were high: mean sequence divergences were 1.1% (range: 0-2.3%) for COI and 5.3% (0.8-9.8%) for CR in *T. japonica,* and 1% (0-2.3%) for COI and 5.4% (1.5-9.5%) for CR in *T. formosana.* These values were comparable to those between the two taxa: 1.1% (0-2.5%) and 5.5% (1.2-9.4%) for COI and CR respectively, indicating little genetic differentiation between the two taxa. The two taxa were clearly distinct from the outgroup taxa *T. squamosa* and *T. kuroshioensis*, which diverged from the COI sequences of *T. japonica* and *T. formosana* by about 20%. Control region sequences were too divergent between ingroup and outgroup taxa (with large segments of base insertions/deletions) to be aligned properly. Thus CR sequences from *T. squamosa* and *T. kuroshioensis* were excluded from the NJ analysis.

4.3.2 Phylogenetic and population genetic analysis

Neighbour-joining analysis of the two genes revealed that individuals from different populations of *T. japonica* (including both grey and purplish-grey color forms) and *T. formosana* intermingled at the terminal nodes. Trees are not shown due to poor resolution among internal nodes. For the COI tree, populations clustered to form a monophyletic group with respect to the outgroup taxa with high bootstrap value (100%). A haplotype network for COI sequences revealed a similar result with individual haplotypes randomly distributed within the network without any clustering with respect to taxa (Fig. 4.2) or localities (Fig. 4.3). Most haplotypes were only found in single individuals. One haplotype was shared by two individuals each of *T. japonica* and *T. formosana*. The haplotype network for the control region sequences (not shown) was inconclusive as a result of high variability and high levels of

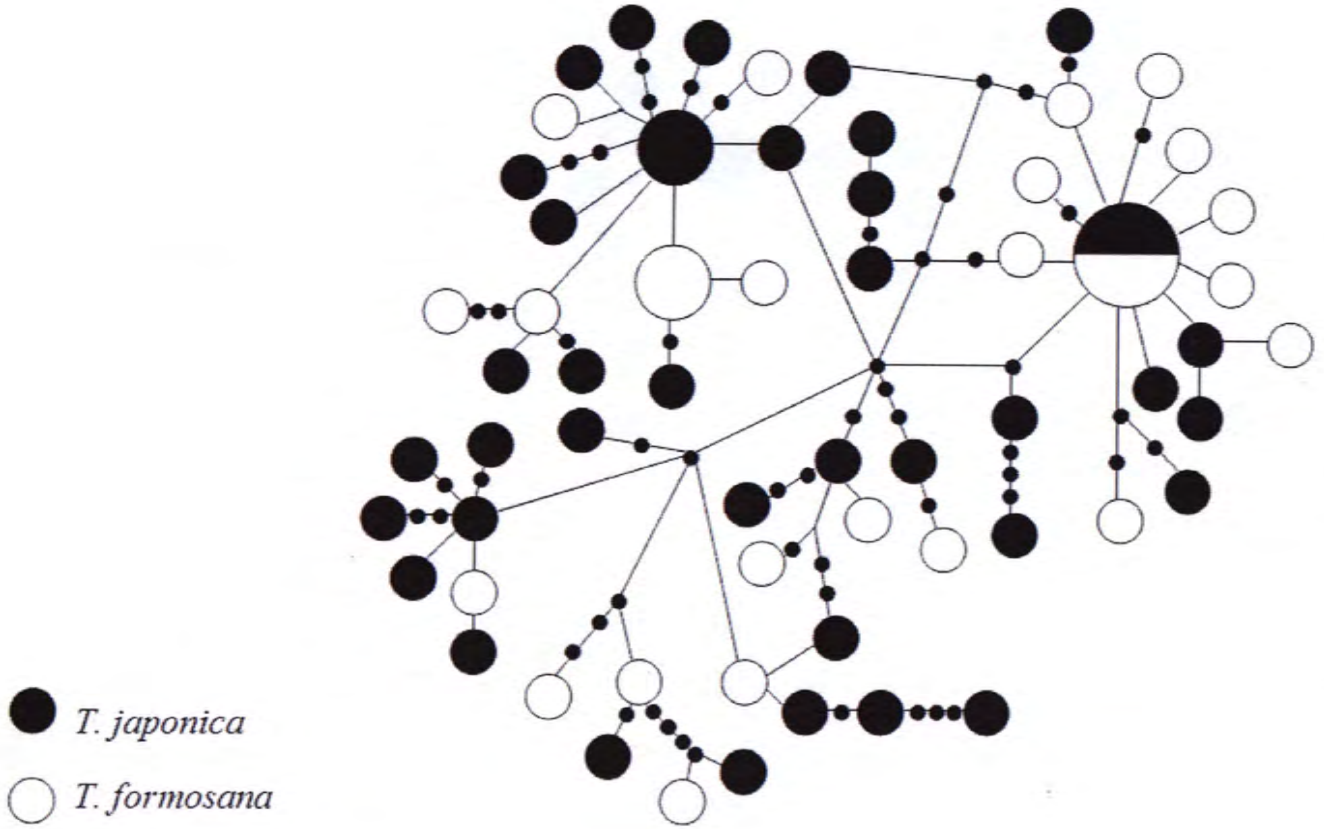


Fig. 4.2 COI haplotype network of *T. japonica* and *T. formosana*. Nodes along each branch designate the number of base differences among haplotypes. Patterns within the circles denote taxon; and the area of the circles corresponds to the number of individuals matching the particular haplotype (range 1-4).

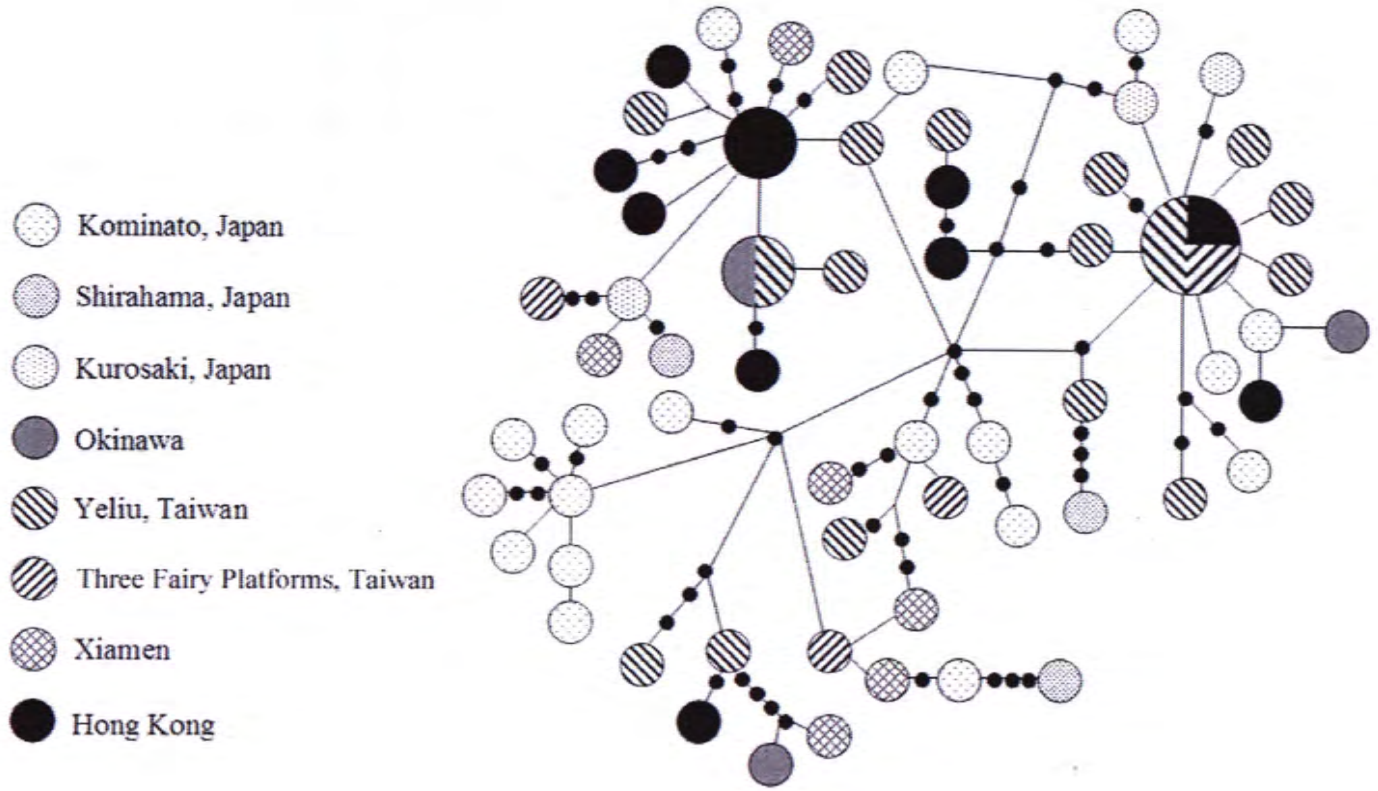


Fig. 4.3 COI haplotype network of *T. japonica* and *T. formosana*. Nodes along each branch designate the number of base differences among haplotypes. Patterns within the circles denote locality; and the area of the circles corresponds to the number of individuals matching the particular haplotype (range 1-4).

homoplasy among haplotypes.

AMOVA analysis revealed no significant genetic differentiation between the two color forms of *T. japonica* (Φ_{CT} <0, P = 0.66). Thus, they were combined as a single population in subsequent analyses. AMOVA (Table 4.2) for the two species revealed no significant genetic differentiation among them. Less than 2.5% of the variation was contributed by differences among the two taxa whereas most of the variation (>94% in COI and CR) was attributed to variation within populations. COI data did not show significant population structuring within two species. However, AMOVA of CR found weak, but significant genetic differentiation among populations within two taxa (Φ_{SC} = 0.034; P = 0.001). The control region was five times more variable than COI in these taxa (mean sequence divergence ~5.5% vs. ~1%); thus it provided greater utility in resolving population structure. A pairwise F_{ST} comparison among different populations (Table 4.3) revealed that the two Japan *T. japonica* populations were genetically differentiated from the other *T. japonica* populations and *T. formosana* populations. There was no significant structuring detected among populations in southern locations (Taiwan and Hong Kong) for both *T. japonica* and *T. formosana* indicating there might be a genetic break between northern and southern. The number of individuals analyzed for *T. formosana* from Japan is too little to provide further evidence for the break.

Table 4.2 Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *T. japonica* and *T. formosana*.

Molecular marker	Source of variation	df	% variation	Φ	*P* value
COI	Among species	1	0.21	$\Phi_{CT} = 0.022$	0.556 ± 0.0049
	Among populations within species	9	2.24	$\Phi_{SC} = 0.022$	0.152 ± 0.0033
	Within populations	64	97.56	$\Phi_{ST} = 0.024$	0.136 ± 0.0035
Control region	Among species	1	2.38	$\Phi_{CT} = 0.024$	0.086 ± 0.008
	Among populations within species	10	3.29	$\Phi_{SC} = 0.034$	0.001 ± 0.001
	Within populations	108	94.33	$\Phi_{ST} = 0.057$	0.000 ± 0.000

Table 4.3 Pairwise F_{ST} values for three populations of *T. japonica* and *T. formosansa* based on control region. *P* values: * = $P < 0.05$, ** = $P < 0.01$. See Table 4.1 for abbreviation for populations.

	J-HK	J-XM	J-BA	J-WA	J-KO	F-KT	F-TF	F-BA	F-OK	F-KU	F-WA
J-HK	-										
J-XM	0.059	-									
J-BA	-0.028	-0.019	-								
J-WA	0.054*	0.034	0.119*	-							
J-KO	0.121**	0.045	0.197*	-0.002	-						
F-KT	-0.004	0.042	0.009	0.027	0.105**	-					
F-TF	-0.025	0.050	0.023	0.035	0.120**	-0.027	-				
F-BA	-0.005	0.113*	0.089	0.080*	0.158**	-0.008	-0.040	-			
F-OK	0.043	0.012	-0.004	0.108*	0.179**	0.017	0.045	0.070	-		
F-KU	0.003	0.133	0.115	0.144*	0.261*	-0.010	-0.067	-0.039	0.088	-	
F-WA	0.004	0.017	0.014	-0.001	0.044	-0.004	-0.024	0.007	0.042	-0.008	-

4.4 Discussion

4.4.1 Molecular variation between Tetraclita japonica *and* T. formosana

In the present study, there was little genetic differentiation among *Tetraclita japonica* and *T. formosana* in their distribution range. The two molecular markers we employed are highly variable. COI has been widely used in delimiting species status and phylogeny in barnacles (Wares 2001; Chan et al. 2007a,b) and other crustaceans (Knowlton 2000; Tsoi et al.. 2005), and the control region is more variable and informative in resolving fine-scale population structuring (Chu et al. 2003; Bernardi and Vagelli 2004). Yet both markers revealed no significant genetic differentiation between the two taxa under study. Our results are congruent with a previous study based on a few individuals which showed only 1.2% COI divergence between the two taxa (Hasegawa et al. 1996). This value overlaps with those of intraspecific sequence divergence in this study, so that it may represent a difference among individuals rather than between taxa. The two taxa also shared common nuclear ITS1 genotype. The present genetic analysis provides no evidence for reproductive isolation between *T. japonica* and *T. formosana*. Moreover, parallel study by Dr B.K.K. Chan (Academia Sinica) based on morphometric analyses on opercular plates also revealed that the two taxa are morphologically very similar (Tsang et al. 2007). The only diagnostic difference between the two was only observed in the color of the parietes and opercular plates. Thus, the two taxa probably represent two color morphotypes/subspecies of *T. japonica.*

4.4.2 Population structure in East Asia

F-statistics indicates that marked genetic structure in *T. japonica/T. formosana* between the northern (Japan) and southern region (Taiwan, and Hong Kong) in East Asia. Oceanographic patterns and geographical distance between the taxa might account for the observed low level of gene flow between the two regions. *Tetraclita japonica* was abundant in the southeastern coast of China and Japan but not common in Taiwan and Okinawa (Chan, unpublished data). The purplish-grey and grey forms of *T. japonica* are considered to be as color morphotypes of the same taxon because they co-exist in high abundance in Honshu, Japan. This is also consistent with the original description of *T. japonica* by Pilsbry (1916), who stated that color of the parietes varies from purple to grey. *T. formosana* was only abundant in Taiwan although it exists in low abundance in Japan and Okinawa. Larval development for *T. japonica* is about 14 days (Chan 2003) and the long geographical distance between Japan and the southern populations may limit gene flow between the two regions. The Okinawa islands, which are located between Japan and Taiwan, have only a low abundance of *T. formosana* and *T. japonica* reflecting a weak connection between the two regions. Strong upwelling in summer months along the coastline of Izu peninsula in Japan, northeastern coastline of Taiwan and the Okinawa Island (Ito et al.. 1995; Ishizaka et al. 1992; Lee and Chao 2003) may further block larval transport along latitudinal gradient and hence serves as a barrier to gene flow between northern and southern populations. However, this population structure has to be investigated by further study due to the high haplotype and nucleotide diversity compared to the relatively small number of populations and individuals examined here (see Chapter 5).

4.4.3 Geographical variation in parietes color: environmental or genetic?

The lack of mtDNA and morphological differentiation between *Tetraclita japonica* and *T. formosana* leads to the question of their species status. Although our data provide no support for the occurrence of two reproductively isolated species, we could not completely rule out the possibility that the two taxa represent two recently diverged species, as there might be retention of ancestral polymorphism in mtDNA because of incomplete lineage sorting. This phenomenon has been well documented in cichlid fishes from Lake Malawi (Moran and Kornfield 1993; Parker and Kornfield 1997) which underwent rapid radiation in less than a million years. However, this scenario seems unlikely for the two taxa in the present study. Since lineage sorting is more rapid in genes with higher mutation rates (Palumbi et al. 2001), one would expect that the gene tree constructed using the more rapidly evolving control region would reveal more complete lineage sorting; thus this marker would exhibit a higher level of differentiation between the two taxa compared with COI. However, the control region did not improve resolution in the gene tree and showed similar levels for Φ_{CT}-values as the COI data, even though it is nearly five times more variable than COI in the present study. Hence, incomplete lineage sorting is not a good explanation for the lack of genetic differentiation between *T. japonica* and *T. formosana.*

Based on these results, *T. japonica* and *T. formosana* probably represent two color morphotypes of the same species with different geographical distribution. Variations in the local environment might have exerted divergent selection pressures leading to different color either through inducing phenotypic plasticity or fixing of

selectively advantageous alleles. Barnacles often exhibit phenotypic plasticity in response to local selection pressures. For example, the rocky shore barnacle *Chthamalus anisopoma* develops different parietes shapes in response to predation pressure from the snail *Acanthina angelica* (Lively 1986). The coral barnacle *Cantellius* shows a high degree of morphological variation in association with different host species (Mokady et al. 1999) with little genetic differentiation (Mokady et al. 1999, 2000). *Tetraclita japonica* exhibits different cirral length in response to wave exposure (Chan and Hung 2005). In the present study, *T. japonica* and *T. formosana* have different geographical distributions and parietes colors which could represent phenotypic plasticity in response to differing local selection pressures after settlement. The selective advantages of having different parietes colors are not known. Variation in color of parietes may affect the absorbance of solar radiation and thus the body temperature of barnacles (see Achituv and Borut 1975), directly bearing on tolerance to heat stress during summer (Chan et al. 2007c). However, there is no evidence that *Tetraclita* can develop different parietes colors in response to environments after settlement. Further studies are needed to provide more information on color development and its ecological significance in barnacles.

Alternatively, color differences between *Tetraclita japonica* and *T. formosana* may be a result of genetic hitchhiking. *Tetraclita japonica* and *T. formosana* may represent two ecotypes of a single species which are adapted to different environments with color controlled by inheritable genetic factors rather than environmental variables. The heterogeneous environment exerts divergent selection pressure on the barnacles of a wide distribution range. Under this scenario, phenotypic differentiation is fixed in different alleles despite the persistence of gene

flow that homogenizes most of the genome, including the selectively neutral mitochondrial genome (Wilding et al. 2001; Campell and Bernatchez 2004). The different colors offer no adaptive advantage to the barnacles so that the gene(s) affecting color may not be directly under selection itself, but could be linked to genes that are adaptively advantageous and fixed in local populations. Individuals with genotypes maladapted to a local environment would be selected against by strong post-settlement selection. As a result, an asymmetric distribution of two ecotypes in different regions appears. This divergent selection on traits between populations in contrasting environments may directly or indirectly lead to reproductive isolation and eventually emergence of new species (Schluter 2001).

In conclusion, the present study suggests that *Tetraclita japonica* and *T. formosana* are probably different color morphotypes of the same species exhibiting different geographical distributions. Geographical separation of the two color morphotypes could be the result of a developmental phenotypic response to a heterogeneous environment or the product of genetic hitchhiking by post-settlement selection on local adaptive genotype. Further analysis on factors affecting the geographical separation of color morphotypes should involve a large number of nuclear markers as in amplified fragment length polymorphism (AFLP). AFLP is a multilocus approach that has been demonstrated to significantly improve resolution in elucidating phylogeny of closely related species (Albertson et al.. 1999), as well as identifying adaptive divergence among populations or morphtypes (Wilding et al.. 2001; Bonin et al. 2006). Resolving the relationship between *T. japonica* and *T. formosana* would provide valuable information on the interaction between environmental factors and phenotypic responses on the rocky shore. This also

provides insight into the evolution of *Tetraclita* and the phylogeography of intertidal biota in East Asia.

Chapter 5

Genetic differentiation and hybridization between *T. japonica* and *T. formosana* revealed by amplified fragments length polymorphism analysis: incipient speciation in glaciation refugia?

5.1 Introduction

In the northwestern Pacific region, a series of marginal seas separate the Asian continent from the Pacific Ocean (Fig. 5.1). During Pleistocene glacial cycle, the lowering of sea level exposed the East China Sea Shelf that moved the shoreline seaward over thousands of kilometers. As a result, Taiwan was fused with the mainland and Japan was also connected to the mainland by land bridges (Xu & Oda 1999). Although this facilitated the migrations of terrestrial and some freshwater fauna between the regions, the land bridges acted as effective barriers to the dispersal of marine taxa. The glaciated habitats forced the marine biota to survive only in fragmented ice-free refugia. Genetic differences accumulated in rudiment populations during the long-term isolation, which provided a driving force for diversification and speciation. Marine organisms persisted in the isolated marginal seas would differentiate into several lineages (Liu et al. 2006a, b, 2007) and new species formation was also observed (Higuchi & Goto 1996; Yamada et al. 2001). The coast of China had undergone more severe fluctuations compared to the Pacific coast of Japan and thus changes in sea level are expected to have greater impact on species there (Xu & Oda 1999). Organisms might be able to persist in Japan in

addition to temperate southern refugia that rescue a large number of species (Amano 2004; Liu et al. 2006a) and the allopatric isolation between these refugial populations are expected to have genetic consequences (Hewitt 2000, 2001, 2004). Thus NW Pacific is an interesting region in studying how organisms adapted and survived through such environmental fluctuations and diverged in different selection regimes. Yet only limited studies have been reported (Liu 2006a, b, 2007), and the interaction between glaciations and speciation remain largely unexplored in this region.

Intertidal barnacles provide an excellent model organism for studying the effect of environmental fluctuations on population differentiation of marine organisms. Adult stages of barnacles are dominant space occupier in the intertidal rocky shore that plays an important role in ecological assemblage (Remier 1976; Bertness et al. 1998). The sessile adult stage that only settle on suitable substratum (Berntsson et al. 2004), making the barnacles sensitive to changes in the coastal habitat. Their dispersal is restricted in the planktonic larval stage that mainly being passively carried by ocean current so that the connectivity among populations reflects the ocean current pattern.

Tetraclita species are dominant intertidal barnacles in NW Pacific and *Tetraclita japonica* and *Tetraclita formosana* are two common taxa in this region. They were originally described as two subspecies of *Tetraclita squamosa* but were later considered to be two distinct species by Yamaguchi (1987) and Hasegawa et al. (1996) based on allozyme and mitochondrial COI analysis on a limited number of samples. However, studies on samples over their geographical range using sequence analysis of mitochondrial COI, control region and nuclear ITS1 demonstrated that

the two taxa are genetically distinct from *T. squamosa* while they are not differentiated (see Chapters 3 and 4). Moreover, recent study on opercular plate morphology revealed no difference between two taxa (Tsang et al. 2007) leading to uncertainty on their species validity.

However, the two taxa clearly differ in parietes color and geographical distribution. *T. japonica* usually has grey parietes and dominates the coast of southern China, Japan and part of Korean Peninsula and is occasionally also found in Taiwan (Fig. 5.1). There is also a purplish-grey parietes form in Japan (Fig. 1.1A). On the other hand, *T. formosana* with pink parietes is highly abundant in eastern Taiwan, and also found in Ryukyu Islands but rare in Japan and absent in southern China. The two taxa thus exhibit parapatric distribution and rarely coexist in high abundance. Yet it remains uncertain whether the lack of genetic divergence of the two taxa indicates that they represent two incompletely sorted lineages or two conspecific color morphotypes. If the different colors reflect genetic differentiation, the two taxa should have been diverged very recently, most probably in the Pleistocene. Under this scenario, they might have evolved in allopatric glaciation refugia and then underwent postglacial range expansion. They might then hybridize in the contact zone as shown in many other organisms which have diverged during the Pleistocene (e.g. Hewitt 2001; Redenbach & Taylor 2002; Fraser & Bernatchez 2005; Gum et al. 2005; Barluenga et al. 2006). Introgression combined with the absence of sufficient time for lineage sorting result in lack of genetic differentiation in the mtDNA and nuclear loci analyzed. The purplish-grey form of *T. japonica* in Japan, where the two taxa occur in sympatry, might be color intergrades of the typical pink and grey color of the two taxa resulted from admixture of two diverged

genome. In contrast, the different colors could be a phenotypic response to different local selection pressure which is not uncommon in many barnacle species (Mokady et al. 2000; Appelbaum et al. 2002).

To assess the overall genomic divergence between *T. japonica* and *T. formosana*, analysis of a large number of independent loci is essential. As the individual gene genealogies are subject to stochastic events and the time required for them to reach monophyly depends on many factors, such as demographic history of the organism, many recently diverged taxa only differ in allele frequencies rather than showing monophyly in gene tree constructed from a few markers (Moritz 1994; Wiens & Servedio 1998). Reticulate evolution across the young species boundary would make the issue even more complicated (Nichols 2001). The AFLP technique (Vos et al. 1995) enables us to obtain a large number of loci without prior knowledge of genome of the target organism. Together with Bayesian based statistical methods, we can detect the differentiation among evolutionarily young taxa (Mock et al. 2002; Spaulding et al. 2006) and the occurrence of admixture and introgression (Gompert et al. 2006).

In the present study, I applied AFLP to test the two alternative hypotheses: (1) the two taxa are genetically diverged during the Pleistocene, or (2) they are two genetically homogenous color morphotypes that are products of local selection pressure.

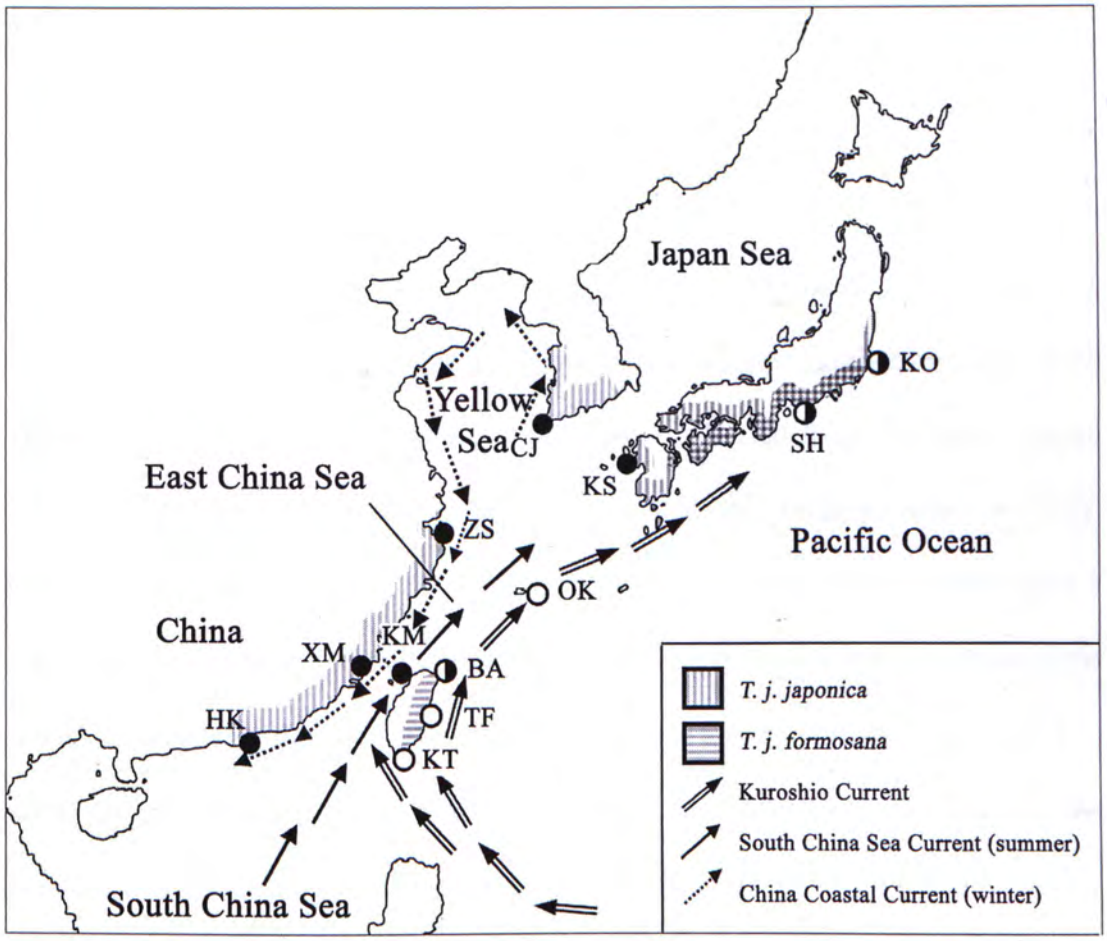


Fig. 5.1 Schematic map of NW Pacific showing the distribution of *Tetraclita japonica* (vertical shading) and *Tetraclita formosana* (horizontal shading) and sampling localities. Open and closed circles denote sampling sites of *T. japonica* and *T. formosana*, respectively. Semi-closed circles represent sites from which both subspecies were collected. See Table 5.1 for abbreviations of populations. Arrows denote the directions of ocean currents.

5.2 Materials and Methods

5.2.1 Sample collection

Barnacles were collected from nine geographical localities for *T. japonica* and six for *T. formosana* (Fig. 5.1, Table 5.1). In Japan Honshu, *T. japonica* exist as two color forms (purplish-grey and grey) while they are grey in other localities. Both purplish-grey and grey forms of *T. japonica* were collected from Shirahama, Japan while only individuals of the grey form could be obtained from the other localities. Only adult barnacles were collected in order to confirm the parietes color. Only a total of about 20 individuals of *T. formosana* could be collected from Japan locations due to its low abundance on the shore. The samples were collected from 2003 to 2006 through the collaboration with Dr BKK Chan (Academia Sinica) and the assistance of several colleagues (see acknowledgements).

5.2.2 AFLP analysis

Total genomic DNA was extracted from whole soft tissue of individual barnacles using the commercial QIAamp Tissue Kit (QIAGEN). AFLP analysis was carried out following Vos et al. (1995) with minor modifications. 100 ng genomic DNA was digested with *Eco*RI and *Mse*I, and ligated with synthetic adapters. After preselective amplification using preselective primers with one selective base (*Eco*RI + A and *Mse*I + C), fragments were amplified in a second round of PCR using selective primers of three selective bases with the *Eco*RI primers fluorescently labeled. We initially tested 21 primer combinations, of which three (E-ACC+M-CAA, E-AAG+M-CTT, E-AGA+M-CTA) yielded informative banding patterns that were easy to score. These primer sets were then used in the analysis of all individuals. The

Table 5.1 Sample localities, abbreviations, sample sizes (*N*), and genetic diversity of *Tetraclita japonica japonica* and *Tetraclita japonica formosana* populations. H_E denotes the unbiased expected heterozygosity.

	Sampling locality	Abbreviation	*N*	% polymorphic loci	H_E	S.E.
T. j. japonica (grey)	Hong Kong, China	J-HK	27	76.6	0.2596	0.0119
	Xiamen, China	J-XM	11	78.0	0.2750	0.0122
	Kinmen, China	J-KM	26	73.7	0.2485	0.0117
	Putou Shan, Zhoushan, China	J-ZS	24	74.2	0.2412	0.0118
	Badoutz, Taiwan	J-BA	4	68.9	0.2805	0.0119
	Cheju, Korea	J-CJ	28	68.9	0.2238	0.0120
	Amakusa, Kyushu, Japan	J-KS	23	80.9	0.2657	0.0117
	Shirahama, Wakayama, Honshu, Japan	J-SH	19	76.1	0.2627	0.0117
	Kominato, Boso peninsula, Honshu, Japan	J-KO	25	77.0	0.2600	0.0114
T. j. japonica (purplish-grey)	Shirahama, Wakayama, Honshu, Japan	P-SH	22	77.0	0.2574	0.0121
T. j. formosana	Kenting, Taiwan	F-KT	23	73.7	0.2286	0.0119
	Three Fairy Platform, Taiwan	F-TF	21	71.8	0.2273	0.0119
	Badoutz, Taiwan	F-BA	36	75.1	0.2451	0.0116
	Okinawa, Japan	F-OK	28	74.6	0.2499	0.0119
	Shirahama, Wakayama, Honshu, Japan	F-SH	22	88.0	0.2931	0.0109
	Kominato, Boso peninsula, Honshu, Japan	F-KO	2	52.6	0.2339	0.0127

resulting amplicons were run on a sequencing gel using an ABI 3100 automated sequencer with a ROX400 (ABI) internal size standard. GENOGRAPHER v1.6 (Benham 2001) was used to visualize and score the banding pattern. Only fragments between 50-400 base pairs that could be scored unambiguously were scored. They were scored as either present (1) or absent (0).

5.2.3 Statistical data analyses

As hybridization would possibly lead to linkage disequilibrium, the allele frequencies at each locus were estimated using a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999) implemented in AFLP-SURV 1.0 (Vekemans 2002). The estimates of allele frequencies were used to calculate the percentage of polymorphic loci and unbiased expected heterozygosity (Nei 1973) to determine the genetic diversity within populations and taxa. A pairwise population genetic distance matrix was constructed based on Nei's (1978) distance using AFLP-SURV 1.0. A neighbor-joining (NJ) tree was constructed based on the distance matrix using the NEIGHBOR program implemented in PHYLIP 3.65 (Felsenstein, 2004). The strengths of internal nodes were assessed by bootstrapping over all loci with 1000 pseudo-replicates generated by AFLP-SURV 1.0 and the bootstrap consensus tree was computed using CONSENSUS in PHYLIP 3.65. To avoid any departure from the assumption of Hardy-Weinberg equilibrium that would lead to bias in NJ analysis and to visualize the clustering of individuals based on genetic similarity, we conducted principal component analysis (PCA) on the allele frequencies across all loci for the samples using PRIMER 6 (Plymouth Routine in Multivariate Analysis).

Hierarchical structuring genetic variations among species, color forms and populations were estimated using analysis of molecular variance (AMOVA; Excoffier et al.. 1992) as implemented in ARLEQUIN version 3.0 (Excoffier et al. 2005). Three different partitions of AMOVA were analyzed. We first investigated the variation between the two color forms of *T. japonica* from Japan and within each form. As population structuring was observed within *T. japonica* (see Results), only samples from Japan and Korea were included in this partition. The second partition investigated variation between the two species and within each species; and finally, we determined the variation partitioned between geographical regions within species. Previous study based on mitochondrial control region sequences revealed population differentiation between *T. japonica* populations from Japan and China (see Chapter 4). Thus, we also investigated the differentiation between the northern (Korea and Japan) and southern (China and Taiwan) samples of *T. japonica*. Population differentiation of *T. formosana* was not analyzed due to the limited number of populations examined and the occurrence of admixture in Japan populations (see Results).

We tested the presence of admixture between individuals using a model-based Bayesian approach as implemented in STRUCTURE 2.1 (Pritchard et al. 2000). This analysis determined the number of populations (genetic clusters, K) without prior information of the origin of individuals. We performed the analysis under a model assuming admixture and correlated allele frequencies between groups. We tested K from 1 to 10 populations with 10 iterations (50 000 burn-in, 250 000 MCMC replicates in each run) to test for the consistency of the run. The optimal value of K was selected using an *ad hoc* statistic ΔK based on the rate of change in

the log probability of data (Evanno et al. 2005). For the selected value of K, we assessed the average proportion of membership (admixture coefficient, q) of the samples to the inferred clusters.

5.3 Results

5.3.1 AFLP pattern and genetic diversity

The three primer pairs generated a total of 209 AFLP bands of which 204 (97.6%) were polymorphic. 196 (93.8%) and 200 (94.4%) bands were polymorphic in *T. japonica* and *T. formosana*, respectively. The genetic diversity was high in all populations (Table 5.1) except the *T. formosana* population from Kominato, Japan for which only two individuals were analyzed. There were no diagnostic bands (i.e., with fixed differences) between the two taxa.

5.3.2 Genetic variation between the two species and among different populations

In the NJ tree (Fig. 5.2), all but one *T. formosana* populations clustered together as a clade with a bootstrap (BP) support of 100. The three populations from Taiwan (F-BA, F-TF and F-KT) grouped together with 100% bootstrap support indicating certain degree of genetic differentiation between Okinawa (F-OK) and Taiwan populations, while Shirahama, Japan population (F-SH) was most distantly related to the other populations in this clade. All *T. japonica* samples (including both grey and purplish-grey forms) grouped together formed another clade (BP = 100) which also included *T. formosana* from Kominato, Japan (F-KO). This clade

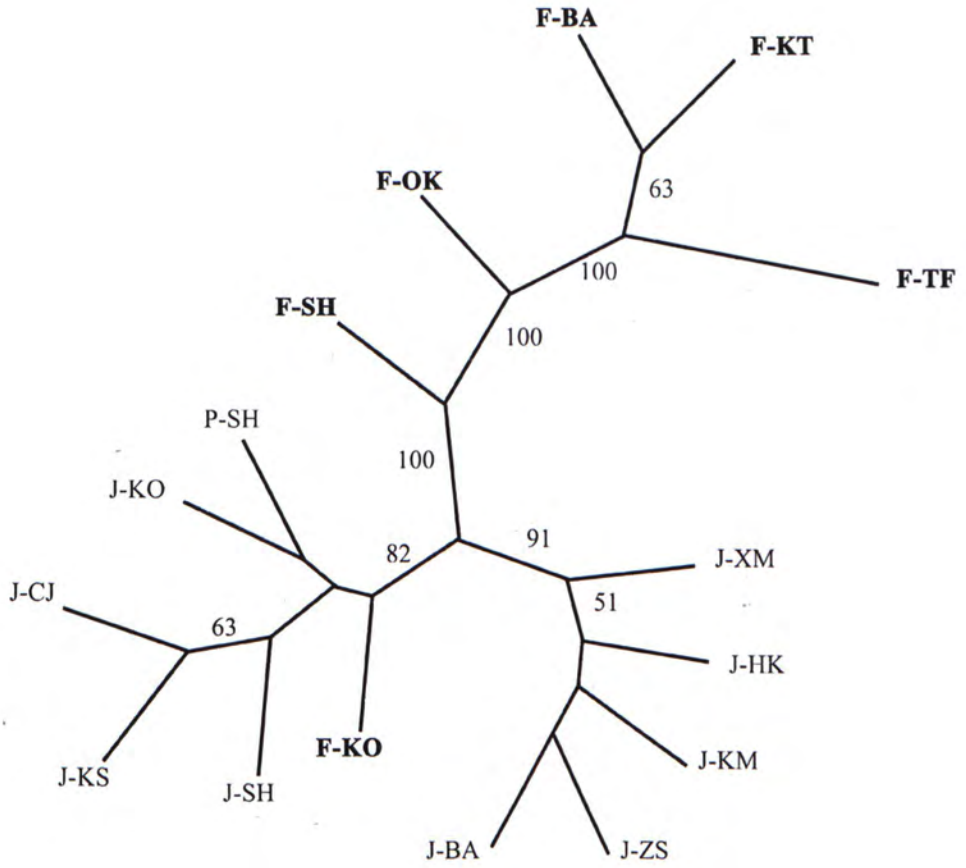


Fig. 5.2 Neighbor-joining tree of Nei's distance among *Tetraclita japonica* (J) and *Tetraclita formosana* (F) populations. Bootstrap values over 50 are shown next to the corresponding branches. Populations of *T. formosana* in bold. See Table 5.1 for abbreviations of populations.

consisted of two highly supported clusters. The first one included populations from southern China (J-HK, J-XM, J-KM and J-ZS) and Taiwan (J-BA) (BP = 91) while the other contained samples from Japan (J-KO, J-SH and J-KS), Cheju, Korea (J-CJ) as well as the purplish-grey form of *T. japonica* from Shirahama, Japan (P-SH) and *T. formosana* from Kominato, Japan (F-KO) (BP = 82). Result of PCA (Fig. 5.3) on individual basis was largely congruent to that of NJ analysis. The samples were divided into two nearly non-overlapping clusters with one containing all *T. japonica* and the other consisting of all *T. formosana* individuals with the exception of some *T. formosana* individuals from Japan (see below). The first principal axis explained 18.3% of the total observed difference which represented the genetic differentiation between the two taxa. *T. japonica* samples were subdivided into two groups, one consisting of individuals from Japan and Korea, and the other including all those from mainland China and Taiwan. This population differentiation contributed to 4.0% of the total differentiation along the second principal axis. The purplish-grey *T. japonica* individuals were mixed with the other grey individuals from Japan. *T. formosana* from Taiwan grouped together and overlapped a bit with the Okinawa population, but individuals from Kominato and Shirahama of Japan did not form a single cluster. Some grouped with *T. formosana* from Okinawa, but many of them appeared to be intermediate between *T. japonica* and *T. formosana*, and a few of them were even genetically more similar to *T. japonica* than to the other *T. formosana* individuals. Thus, some *T. formosana* from Japan could represent hybrids of the two taxa or the products of backcross of later generations.

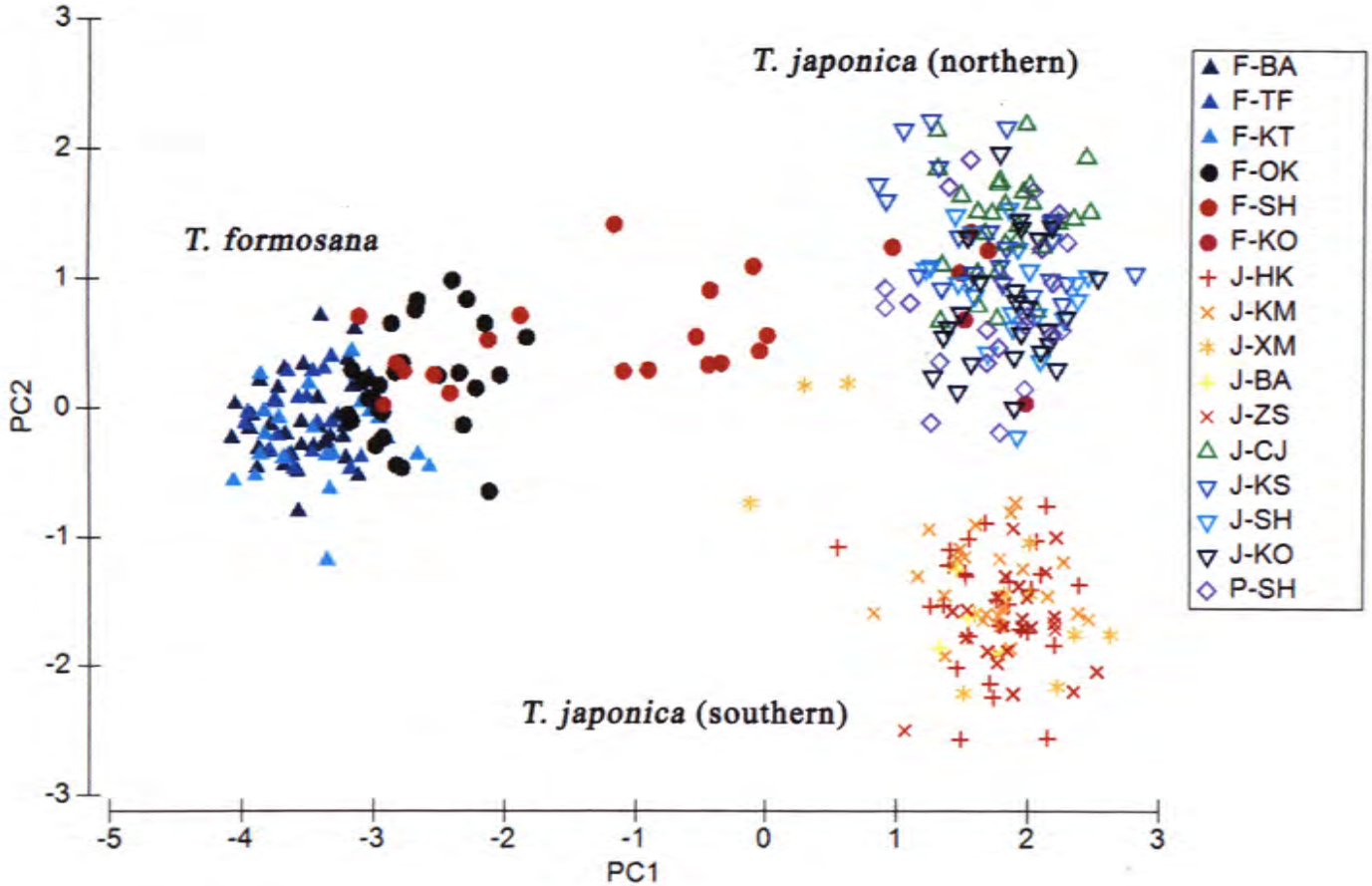


Fig. 5.3: Principal component analysis (PCA) based on allele frequencies of 209 AFLP markers for 341 individuals of *Tetraclita japonica* and *Tetraclita formosana*. The plot shows the first and second principal axes, which accounted for 18.3% and 4.0% of the total variation, respectively.

AMOVA revealed no significant genetic differentiation between the two color forms of *T. japonica* (negative and non-significant Φ_{CT}, Table 5.2A). Thus we combined the individuals of the two forms from the same sampling locality as a single population in subsequent AMOVA analysis. There was a significant and large genetic differentiation between the two species ($\Phi_{CT} = 0.267$, $P < 0.001$; Table 5.2B). As there was putative admixture between the two species within the Japan *T. formosana* individuals revealed by PCA and NJ analysis, we excluded the *T. formosana* from Japan and analyzed the data again. The genetic differentiation between the two species became more pronounced ($\Phi_{CT} = 0.314$, $P < 0.001$; Table 5.2C). When considering population structuring within species, there was also a clear genetic differentiation between the northern and southern populations of *T. japonica* (Φ_{CT} =0.098, $P = 0.004$; Table 5.2D), supporting the results of NJ analysis and PCA. There was also some differentiation in *T. formosana* between Okinawa and the three Taiwan locations based on pairwise F_{ST} comparison, but it was not significant when partitioned into two groups in AMOVA (data not shown).

5.3.3 Assessing population clustering by Bayesian analyses and admixture analyses

The presence of two clusters best explained the AFLP data based on the ΔK value (Fig. 5.4). Under the admixture model of $K = 2$, we estimated the proportion of membership, q (admixture coefficient) of individuals to the inferred clusters. We set the threshold $q > 0.8$ to assign the individual genotypes to cluster I (*T. formosana*) and individuals with $q < 0.2$ to cluster II (*T. japonica*), so that individuals would be of mixed ancestry when q ranged from 0.2 to 0.8. All but one *T. formosana* individuals from Taiwan and Okinawa had $q > 0.8$, with a mean >0.95 (Fig. 5.5).

Table 5.2 Results of hierarchical analysis of molecular variance (AMOVA) of AFLP. (A) AMOVA for partition among two color forms of *Tetraclita japonica japonica*. (B) AMOVA for partition among two subspecies. (C) AMOVA for partition among two subspecies with *Tetraclita japonica formosana* in Japan excluded. (D) AMOVA for partition among northern and southern samples of *T. j. japonica*.

(A)

Source of variation	d.f.	Sum of square	Variance component	% variation	Φ	*P* value
Among forms	1	48.701	-0.667	-2.70	Φ_{CT} = -0.027	0.786
Among populations within forms	3	225.421	2.202	8.91	Φ_{SC} = 0.087	<0.001
Within populations	112	2594.852	23.168	93.79	Φ_{ST} = 0.062	<0.001

(B)

Source of variation	d.f.	Sum of square	Variance component	% variation	Φ	*P* value
Among subspecies	1	1687.532	9.740	26.75	Φ_{CT} = 0.267	<0.001
Among populations within subspecies	13	1238.449	3.257	8.95	Φ_{SC} = 0.122	<0.001
Within populations	326	7633.966	23.417	64.31	Φ_{ST} = 0.357	<0.001

(C)

Source of variation	d.f.	Sum of square	Variance component	% variation	Φ	*P* value
Among subspecies	1	1801.556	11.917	31.44	Φ_{CT} = 0.314	<0.001
Among populations within subspecies	11	1013.616	2.907	7.67	Φ_{SC} = 0.112	<0.001
Within populations	304	7016.103	23.079	60.89	Φ_{ST} = 0.391	<0.001

(D)

Source of variation	d.f.	Sum of square	Variance component	% variation	Φ	*P* value
Among regional groups	1	353.336	2.744	9.81	Φ_{CT} = 0.098	0.004
Among populations within regional groups	7	440.718	1.784	6.37	Φ_{SC} = 0.071	<0.001
Within populations	200	4690.702	23.454	83.82	Φ_{ST} = 0.162	<0.001

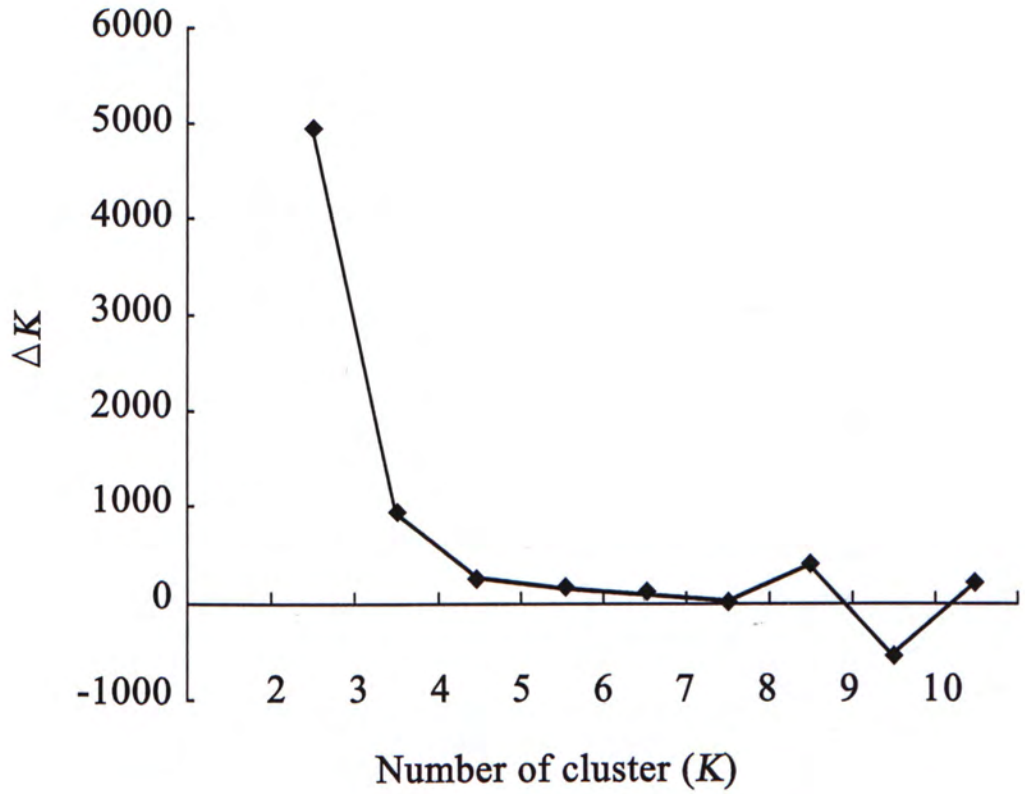


Fig. 5.4: The number of clusters (K) vs. the rate of change in posterior probability (ΔK). The clear maximum at K=2 indicates that two clusters best explain the AFLP data in the present study.

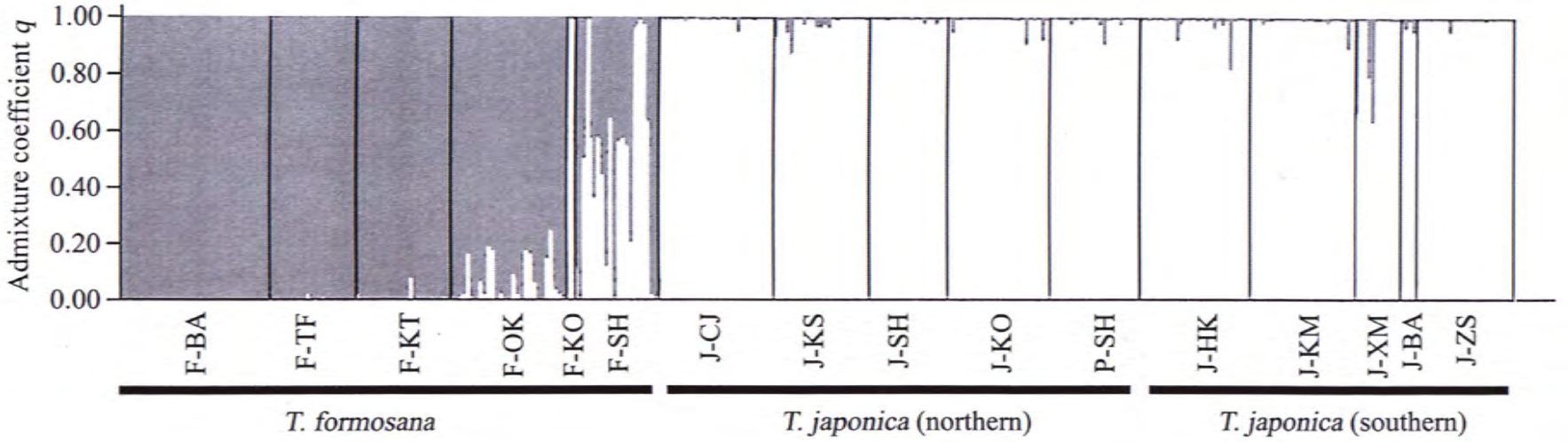


Fig. 5.5: Bar plot of the results obtained from STRUCTURE using K=2. Each individual is represented by a vertical line partitioned into two color segments with length proportional to the admixture coefficients of the two clusters. See Table 5.1 for abbreviations of populations.

Only three *T. japonica* individuals had a q value of > 0.2 but none of the values were >0.35 and the mean value of q over all *T. japonica* samples was <0.05 (Fig. 5.5). However, *T. formosana* from the two localities in Japan had q ranged from 0.01 to 0.98 (mean = 0.51) indicating a mixed ancestry of the *T. formosana* individuals in the sympatric zone of the two species (Fig. 5.5). The presence of *T. formosana* individuals with q between 0 and 0.5 indicated the occurrence of F2 and/or backcross of hybrid individuals. Yet it is not possible to accurately estimate the proportion of hybrid genotypes of different generations to parental genotypes due to the limited number of samples examined.

5.4 Discussion

5.4.1 Genetic differentiation and proposed subspecies designation

Tetraclita japonica and *T. formosana* are genetically highly differentiated. The pink barnacles designated as *T. formosana* are markedly more closely related to one another than to the grey or purplish-grey forms of *T. japonica* in all analyses. The only exception is that some *T. formosana* individuals in Japan appear to be genetically intermediate or more similar to *T. japonica* apparently due to hybridization and subsequent backcross. In contrast, no obvious signals of introgression are detected in the purplish-grey individuals of *T. japonica* although they have been considered to be intermediate in parietes color and suspected to be putative hybrids. They are genetically indistinguishable from the grey form. Our data suggest that difference in parietes color reflects genetic differentiation rather than a phenotypic response to environmental factors. They probably diverged recently and

were not separated for a period sufficient for the development of complete reproductive isolation. The occurrence of hybridization, incomplete lineage sorting, or the mix of both resulted in the lack of differentiation in mtDNA and nuclear ITS1 sequences observed in previous studies (Tsang et al. 2007). Since the two taxa can interbreed, they are not "good" biological species. Therefore, I propose that *T. formosana* should be considered as subspecies of *T. japonica* based on differences in parietes color and geographical distribution.

5.4.2 Evolutionary history of the two species in relation to Pleistocene glaciation

Despite the potential for other forces, including adaptation and ocean current pattern, the divergence of the two species were most likely initiated by vicariance events during the Pleistocene glaciation period. The lowering of sea level during late Quaternary glacial cycle exposed the East China Continental Shelf and the shoreline was moved seaward over thousand of kilometers connecting Taiwan to the mainland (Xu & Oda 1999). This led to dramatic changes in the ocean current pattern (Ujiié & Ujiié 1999; Ujiié et al. 2003) as well as coastal habitat along East China (Wang 1999). The deterioration in rocky shore habitats and the coverage by ice might kill off biota over most of their range. The effect might be even more severe for rocky intertidal species that require a hard substratum making them sensitive to any changes on surface habitats (Wares and Cunningham 2001, Wares 2002). Rudiment populations might survive in ice-free glacial refugia in more temperate southern regions and most of the present day northern communities are widely believed to be the result of postglacial northward range expansion and re-colonization from the south (Hewitt 1999; Dynesius & Jansson 2000).

However, there are increasing evidences for the presence of cryptic northern refugia that retained considerable amount of genetic diversity (Stewart & Lister 2001, Jacobs et al. 2004). The allopatric isolation between southern and northern refugia could have led to genetic divergence and speciation in many North American coastal marine taxa including fishes (Gonzalez-Villasenor & Powers 1990; Hickerson & Ross 2001; Hickerson & Cunningham 2005) and invertebrates (Arndt & Smith 1998; Kyle & Boulding 2000; Marko 2004), including barnacles (Sotka et al. 2004). Similar pattern was also observed in several NW Pacific fishes (Liu et al. 2006a, b, 2007). If *Tetraclita japonica* could also persist in both northern and southern refugia during the Pleistocene, genetic differentiation between these isolated populations could be expected and they possibly emerged as the two species. So it is important to determine whether northern persistence or re-colonization is more likely to have been the case in *Tetraclita japonica* in order to elucidate the possible origins of the two species. Northern populations from postglacial colonization usually experienced demographic bottleneck leading to dramatic reduction in genetic diversity (Hewitt 2000; Lessa et al. 2003). Yet no obvious difference in genetic diversity among the northern and southern populations of *T. japonica* was revealed by AFLP (Table 5.1) and mtDNA analysis (see Chapter 4; Tsang et al 2007). Although we cannot completely rule out the possibility of rapid recovery of genetic diversity in northern populations by high level of gene flow from the south, this seems not likely as there is strong genetic differentiation between northern and southern populations of *T. japonica* in the present study indicating restricted regional dispersal. Thus, the persistence hypothesis appears to be a more parsimonious explanation to the high genetic diversity observed. There was also report on stable northern populations of

the Japanese sea bass *Lateolabrax japonicus* in NW Pacific during glacial periods (Liu et al. 2006a). Moreover, vertical distribution also plays an important role in determining whether an intertidal taxon could persist in the north (Marko 2004; Hickerson & Cunningham 2005). Organisms live in lower shore level are more likely to survive in the north than those inhabit relative higher on the shore as the time exposed to cold air is shorter (Kyle & Boulding 2000; Marko 2004). *T. japonica* and *T. formosana* are mid-shore occupiers so that their exposure time to cold stress in air is relatively shorter. And *T. japonica* is a relatively cold tolerant species as its present distribution extends up to the northern part of Japan (Yamaguchi 1987), suggesting it is likely they could survive in northern refugia during glaciation. All of these evidences support the persistence of *Tetraclita* survivors in the north during the Pleistocene and the two species were originated from vicariance of glaciation refugia.

Although there is a lack of studies on the distributions and locations of glaciation refugia in NW Pacific, the Pacific coast of Japan and eastern coast of Taiwan appear to be two ideal areas for refugia, and thus the origins of the two *Tetraclita* species. Because the sea level continuously fluctuated following the oscillation of glacial and interglacial periods (Imbrie et al. 1992; Lambeck et al. 2002), the East China coast retreated landward when sea level rose, and then expanded seaward again when sea level dropped later. The cycle repeated for several times during the Pleistocene (Imbrie et al. 1992; Lambeck et al. 2002). This caused periodical loss of coastal habitats along the East China coast (Xu and Oda 1999) that would eradicate most intertidal biota. In contrast, changes in areas and configurations along the eastern coast of Taiwan and the Pacific coast of Japan were less drastic (Xu

and Oda 1999) and might provide relatively stable refugia for coastal organisms which maintain stable populations throughout. The coast of Japan was shown to be a shelter for a number of marine fauna during the Pleistocene (e.g. Amano et al. 1996; Amano 2004; Liu et al. 2006a, 2007) which probably included *Tetraclita* as well. Moreover, the present day southern limits of *T. japonica* and *T. formosana* are near southern China and the southern tip of Taiwan respectively (Fig. 1; BKK Chan, unpublished data) and they are excluded by other tropical species further south. Thus, the eastern coast of Taiwan in the Pleistocene was very close to the southern limit today in latitude, and served as the southern refugium. Ancestral populations of *T. japonica* probably persisted in these two allopatric refugia and diverged with *T. japonica* originated from the Japan population and individuals in Taiwan evolved to *T. formosana*.

After the Last Glacial Maximum (LGM; ~18,000 years before present; Voris 2000), the East China coast retreated following retreat of glacier and rise in sea level. Taiwan and Japan separated from the mainland and the stabilized East China coast opened a new area for re-colonization of biota so that organisms underwent rapid demographic and range expansion. The dispersal, and thus colonization, of barnacles are driven by the ocean currents that carry planktonic larvae so that the route of re-colonization and their distribution pattern are largely determined by the oceanographic regime. The Pacific coastlines of Japan, Ryukyu Islands and eastern coast of Taiwan are under the influence of Kuroshio Current which flows northward all year round (Ito et al. 1995; Lee & Chao 2003; Fig. 5.1) so that the larvae of *T. formosana* could be transported northward to reach Okinawa and Japan from Taiwan and settle there. In contrast, the East China coast is under the influence of South

China Sea Warm Current from the South China Sea when the southwest monsoon is prevailing in summer and cannot receive larval supply of *T. formosana* from Taiwan via the Kuroshio Current. A sub-branch of Kuroshio Current enters the Taiwan Strait in the summer but it is very close to the coastline of western Taiwan and thus not affects the coastline of mainland China (Jan et al. 2004). During winter, the northeast winter monsoon winds prevail so that the China Coastal Current is driven to flow southward (Hu et al. 2000; Guan & Fang 2006; Fig. 5.1). This could carry plankton and pelagic larvae along the southern coast of China from the Yellow Sea, East China Sea (Hwang & Wong 2005; Hwang et al. 2006). Although *T. japonica* mainly reproduces during summer, sparse settlement pulses could also be observed in Hong Kong during winter (Chan & Williams 2003, 2004) indicating the occurrence of spawning and dispersal of *T. japonica* in winter. Thus, it is possible that the larvae of winter pulses of *T. japonica* could travel from the Korea peninsula and neighboring areas to colonize the southern coast of China by winter currents. The abundance of *T. formosana* in Japan is too low to supply enough larvae to arrive and recruit in the China coast, leading to the present day distribution of the two species: *T. formosana* is mainly restricted to Taiwan, Okinawa and rarely found in Japan and *T. japonica* occurs in Japan and China coast but not in Taiwan and Okinawa.

The northern limit of *T. japonica* in mainland China is near the mouth of Yangtze River (Fig. 5.1; BKK Chan unpublished data). There is a discontinuity in its distribution between the northern (Japan and Korean) and southern (southern China) populations around the Yellow Sea. The upwelling around the coast of Japan in summer (Ishizaka et al. 1992; Ito et al. 1995; Lee & Chao 2003) and the long distance dispersal would reduce the level of gene flow between two regions, leading

to the observed population structuring among northern and southern *T. japonica* individuals. The larval period of *T. kuroshioensis* is longer than *T. j. formosana* (14-21 days vs. about 7 days; BKK Chan unpublished data), so that the effect of upwelling on dispersal of the former species is less affected.

5.4.3 Hybridization between the two species and population structuring

Avise et al. (1998) surveyed mtDNA divergence between sister species pairs across vertebrates and discovered that most species usually took more than two millions years to reach full speciation and many species separated during the Pleistocene could not achieve complete reproductive isolation. Although this might not be directly applicable to the speciation duration of invertebrates, it is little doubt that the development of reproductive isolation by genetic drift in allopatric refugia requires over millions of years. If the two *Tetraclita* species diverged during Late Quaternary, it is not surprising that they can hybridize in secondary contact. Yet we observed a significant genetic differentiation between the two species in spite of the occurrence of hybridization. The question is which factors contribute to the maintenance of the genetic integrity of the two species.

The oceanographic pattern combined with the biological attributes of the taxa that restricts the dispersal of individuals among the distribution range of the two species might play an important role in limiting the occurrence of hybridization. Although it is hermaphroditic, *T. japonica* mate with neighboring individuals using a long penis to transfer sperm into the mantle cavity of the mate rather than carry out self-fertilization in natural conditions (Murata et al. 2001). This sessile organism,

thus, is only able to copulate with nearby individuals so that hybridization only occurs in locations with settlements of both species. Due to ocean current and larval supply pattern, *T. japonica* larvae rarely reach Okinawa or Taiwan which are under the influence of the Kuroshio Current flowing northward all year round. The few individuals from northern Taiwan examined in the present study were collected a few years ago but we failed to sample any more *T. japonica* in subsequent visits to the same location and surrounding areas. As a result, the individuals previously sampled might be occasional, rare settlements. On the other hand, the Kuroshio Current facilitates the dispersal of marine organisms from Taiwan to Japan so that the two regions share many species in common (Utinomi 1971), including another congeneric barnacle species, *Tetraclita kuroshioensis* (Chan et al. 2007b). Thus, in theory, larval supply of *T. formosana* could reach Japan and provides the major chance that leads to sympatry of the two species. However, the larval development of *T. formosana* only lasts for about one week under laboratory culture conditions (BKK Chan, unpublished data), which is relatively shorter than period of about two weeks in other barnacles (Burrows et al. 1999) including *T. japonica* (Chan 2003). The relatively short pelagic larval duration might not allow the larvae to travel through the long distance between Taiwan, Okinawa and Japan. Moreover, strong upwelling along the coastline of Izu Island of Japan, northeastern coastline of Taiwan and the Ryukyu Islands (Ishizaka et al. 1992; Ito et al. 1995; Lee & Chao 2003) could further block larval transport along the latitudinal gradient. This is supported by the much lower abundance of *T. formosana* in Okinawa than in Taiwan (BKK Chan unpublished data) and the genetic differentiation between Okinawa and Taiwan populations as revealed by AFLP analysis in this study reflecting a restricted level of northward dispersal. The population structuring found in *T. japonica* provides further

evidences to support that the oceanographic pattern around southern Japan serves as an effective barrier to northward gene flow. Only a limited number of *T. formosana* larvae could reach Japan and even fewer of them could successfully compete with the highly abundant and more locally adapted *T. japonica* larvae. As a result, *T. formosana* is only present in very low abundance on the shore of Japan so that the frequency of hybridization is very low compared to pure *T. japonica* cross. Although we are not sure whether the hybrids are less fit than their parents, it would be certain that their low abundance would restrict the number of recruits on the shore so that they could only mate with *T. japonica* even if they could successfully settle. Then the genetic signature of *T. formosana* would soon be diluted and disappeared after a few generations of backcross so that the *T. japonica* sampled in the present study show no obvious signals of introgression.

Our results suggest that the two species probably diverged in historic isolation in different glaciation refugia. Their genetic differentiation is maintained by oceanographic regime that restricts contemporary gene flow, and local adaptation to some extent. Many intertidal species could be found in both Taiwan and Japan under the influence of Kuroshio Current (Utinomi, 1971). Yet there are also several species of endemic marine and estuarine crabs in Taiwan (e.g. *Uca formosensis* and *Eriocheir formosa*; Chan et al. 1995; Shih et al. 1999) indicating that biological attributes of these species could limit their dispersal predicted solely by ocean current pattern. Further studies on a wide array of taxa would help us to elucidate the interplay between vicariance and dispersal that determines the genetic structure of marine biota in NW Pacific. This will not only enhance our knowledge in the phylogeography of this biodiversity rich region, but also reveal how the biological

characters of organisms affect their survival through dramatic environmental changes like glaciation in the past.

Chapter 6 General Conclusion

The genetic differentiation among the three *Tetraclita* species in the East Asian region was studied and a new taxonomic scheme of these species is proposed (see Table 6.1 for a summary).

The green barnacle designated as *Tetraclita squamosa* constitutes a cryptic species complex. The individuals collected from Japan, Okinawa and Taiwan are genetically and morphological differentiated from those in S China and thus represent a new species, *Tetraclita kuroshioensis*. *T. squamosa* distribute along the S China coast while *T. kuroshioensis* is found in NW Pacific and Oceania region. A recent study in our laboratory also indicates the presence of another new species in Singapore (Chan et al 2007b), and individuals in Australia may also be genetically different. Further investigations on samples throughout the Indo-Pacific region are needed to explore the diversity within this species complex and elucidate the actual distribution range of each species.

On the other hand, *T. japonica* and *T. formosana* are found to be genetically indistinct in mtDNA control region, COI and nuclear ITS1. However, the two taxa are genetically highly differentiated as revealed by AFLP analysis. Hybridization occurs in Japan, where the two taxa occur sympatrically, leading to introgression of mtDNA. The low genetic divergence between the two taxa suggests that they diverge recently, probably during the Pleistocene glaciation period. The insufficient time for the development of complete reproductive isolation results in hybridization in their contact zone in Japan. The genetic differentiation is maintained by oceanographic

pattern that restrict gene flow between the two taxa. Based on these results, I suggest that *T. japonica* and *T. formosana* should be considered as two subspecies, *T. japonica japonica* and *T. japonica formosana*, based on their difference in parietes color and distribution pattern. A summary on the taxonomy of *Tetraclita* spp. in East Asia and their geographic distribution is shown in Table 6.1. Comparing this table with Table 1.1 illustrates the major findings of this thesis research.

Table 6.1 A comparison among the three *Tetraclita* species in East Asia.

	T. squamosa	*T. kuroshioensis*	*T. japonica japonica*	*T. japonica formosana*
Parietes color	Green	Green	Grey to purplish grey	Pink
Distribution	South China	NW Pacific and Oceania	South China and Japan	Taiwan, Okinawa and Japan

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